Impact of inhibitors associated with lignocellulosic hydrolysates on recombinant cellulolytic enzymes

by

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DECLARATION

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SUMMARY

Enzymatic hydrolysis contributes a significant cost towards the production of bioethanol and is estimated to comprise 15% of the minimum ethanol selling price. One of the areas of concern during the enzymatic hydrolysis is the non-productive adsorption of enzymes by pretreatment by-products that may lead to the inhibition/deactivation of cellulases. Non-productive adsorption of cellulases onto lignin is mainly driven by hydrophobic interactions and the extent of adsorption varies depending on the hydrophobicity of the lignin. Most fungal cellulases are bimodular with a catalytic domain and a carbohydrate binding domain (CBM) connected by a flexible linker. To achieve high yields of fermentable sugars for subsequent conversion to ethanol, it is desirable to include sugars from both cellulosic- and hemicellulose-rich fractions, which implies the presence of inhibitory degradation compounds during enzymatic hydrolysis. To reduce the enzyme loading for hydrolysis, the inhibitor compounds in lignocellulosic biomass should be reduced to below toxic levels or be removed from hydrolysates.

The first aim of the study was to investigate the role of individual lignocellulose-associated compounds in the inhibition and/or deactivation of the *Talaromyces emersonii* cellobiohydrolase (TeCel7A) fused to the *Trichoderma reesei* carbohydrate binding domain (TrCBM), *Trichoderma reesei* endoglucanase TrCel5A and *Saccharomycopsis fibuligera* β-glucosidase (SfCel3A) cellulases. The second aim was to explore detoxification strategies in the alleviation of the cellulose inhibition. The final aim was to investigate the mechanism(s) involved in the inhibition of cellulases. The impact of selected inhibitor compounds on the hydrolysis of Avicel was also investigated using a combination of TeCel7A-TrCBM and TrCel5A in the presence of Novozyme 188 Cel3A to prevent feedback inhibition by cellobiose. The study revealed that polymeric phenols, such as tannic acid, are strong inhibitors of cellulases, whereas monomeric phenols with aldehyde groups showed a strong inhibition of cellulose with increased contact time. This further confirmed that compounds with increased surface hydrophobicity have a strong inhibition effect.

TrCel7A was shown to be quite resistant to inhibition and only hydroxymethyl furfural (HMF) strongly inhibited this cellobiohydrolase. This selective inhibition of retaining cellulases (TrCel7A), but not inverting cellulases (TrCel5A), was also observed with acetic and formic acid. This suggests that the non-processive nature and groove-shaped active site of TrCel5A allows it to escape non-productive binding to inhibitor compounds through the same

mechanism it employs during cellulose hydrolysis. Further investigation revealed that increasing inhibition was not linked to contact time, but rather ascribed to increased concentration of inhibitor compounds. Detoxification strategies were explored as enhancers of enzymatic hydrolysis and tools to alleviate inhibition in biomass conversion processes. The results indicated that reducing agents (sodium dithionite and sodium sulfite) strongly reacted with coniferyl aldehyde and syringaldehyde, but not tannic acid.

The addition of reducing agents substantially increased the hydrolysis of Avicel containing 10% bagasse pretreatment liquid. Application of the differential scanning fluorimeter (DSF) technique showed that increased concentrations of furans and acetic acid sharply increased unfolding of TeCel7A. This study showed that DSF could be developed as a tool to study cellulase binding, but this will depend on the development of dyes not based on hydrophobic interactions.

OPSOMMING

Ensiematiese hidrolise dra 'n aansienlike koste tot die produksie van bio-etanol by en verteenwoordig na raming 15% van die minimum etanolverkoopprys. Een kwelpunt tydens ensiematiese hidroliese is die nie-produktiewe adsorpsie van ensieme aan neweprodukte van die voorafbehandeling wat tot die onderdrukking/deaktivering van sellulases kan lei. Nie-produktiewe adsorpsie van sellulases aan lignien word hoofsaaklik deur hidrofobiese interaksies gedryf en die omvang van adsorpsie wissel na gelang van die hidrofobisiteit van die lignien. Die meeste swam-sellulases is bimodulêr met 'n katalitiese domein en 'n koolhidraatbindende domein (CBM) wat deur 'n buigsame skakel verbind is. Ten einde 'n hoë opbrengs van fermenteerbare suikers vir daaropvolgende omskakeling na etanol te verseker, is dit wenslik om suikers van beide sellulose- en hemisellulose-ryke fraksies in te sluit, wat die teenwoordigheid van inhiberende afbraakprodukte tydens die ensiematiese hidroliese impliseer. Om die ensiemlading vir hidroliese te verlaag, moet die inhiberende verbindings in sellulose-biomassa tot onder toksiese vlakke verlaag of uit die hidrolisaat verwyder word.

Die eerste doel van die studie was om die rol van individuele lignosellulose-geassosieerde verbindings in die onderdrukking en/of deaktivering van die *Talaromyces emersonii* sellobiohidrolase (TeCel7A) gekoppel aan die *Trichoderma reesei* koolhidraatbindende domein (TrCBM), *Trichoderma reesei* endoglucanase (TrCel5A) en *Saccharomycopsis fibuligera* β-glukosidase (SfCel3A) sellulases te ondersoek. Die tweede doel was om ontgiftingstrategieë vir die verligting van sellulase-inhibisie te verken. Die finale doel was om die meganisme(s) betrokke by die inhibisie van sellulases te ondersoek. Die impak van geselekteerde inhibeerderverbindings op die hidroliese van Avicel is ook met behulp van 'n kombinasie van TeCel7A-TrCBM en TrCel5A in die teenwoordigheid van Novozyme 188 Cel3A ondersoek om terugvoeronderdrukking deur sellobiose te voorkom. Die studie het getoon dat polimeriese fenole, soos looisuur, sterk inhibeerders van sellulases is, terwyl monomeriese fenole met aldehiedgroepe 'n sterk onderdrukking met verlengde kontaktyd met sellulases getoon het. Dit bevestig verder dat verbindings met 'n verhoogde oppervlakhidrofobisiteit 'n sterk onderdrukkingseffek het.

TrCel7A was redelik bestand teen onderdrukking en slegs hidroksielmetielfurfuraal (HMF) het hierdie sellobiohidrolase sterk onderdruk. Die selektiewe onderdrukking van behoudende sellulases (TrCel7A), maar nie omkerende sellulases (TrCel5A) nie, is ook met asynsuur en mieresuur waargeneem. Dit dui daarop dat die nie-prosessiewe aard en groefvormige aktiewe

setel van TrCel5A die ensiem toelaat om nie-produktiewe binding aan inhibeerderverbindings te ontsnap deur dieselfde meganisme wat tydens sellulose-hidroliese gebruik word. Verdere ondersoek het getoon dat verhoogde onderdrukking nie weens kontaktyd was nie, maar eerder die gevolg van verhoogde konsentrasie van inhibeerderverbindings. Ontgiftingstrategieë is ondersoek as versterkers van ensiematiese hidroliese en gereedskap om onderdrukking in biomassa-omskakelingprosesse te verlig. Die resultate het getoon dat reduseermiddels (natriumditioniet en natriumsulfiet) sterk met konifeeraldehied en seringaldehied gereageer het, maar nie met looisuur nie.

Die byvoeging van reduseermiddels het die hidroliese van Avicel met 10% bagasse behandelingsvloeistof aansienlik verhoog. Toepassing van die differensiële skandeer-fluorimeter (DSF) tegniek het aangedui dat verhoogde konsentrasies van furaan en asynsuur die ontvouiing van TeCel7A skerp verhoog het. Hierdie studie het getoon dat DSF as instrument ontwikkel kan word om sellulasebinding te bestudeer, maar dit is onderworpe aan die ontwikkeling van kleurstowwe wat nie op hidrofobiese interaksies gebaseer is nie.

BIOGRAPHICAL SKETCH

Sizwe Innocent Mhlongo was born on the 13th of October 1989 and raised in Durban in the area of Adams Mission. He matriculated in 2006 from KwaMakhutha Comprehensive High School at KwaMakhutha and achieved a distinction with merit. He then enrolled for a Bachelor of Science degree in 2007 at the University of KwaZulu-Natal, majoring in Biochemistry and Microbiology. In 2009 he finished his undergraduate degree and continued for a BSc Honours degree in 2010 in Biochemistry. In 2013 he graduated with a Master of Science degree in the field of Biochemistry at the University of KwaZulu Natal, and enrolled the same year for a Doctor of Philosophy degree in Microbiology at Stellenbosch University.

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PREFACE

This dissertation is presented as a compilation of six chapters and they are written following the style in the *Journal for Microbiology*.

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LIST OF ABBREVIATIONS

USA	United State of America
EU	European Union
WIS	water insoluble solids
DSC	differential scanning calorimetry
DSLS	differential static light scattering
CD	catalytic domain
AFM	atomic force microscopy
BSA	bovine serum albumin
PEG	polyethylene glycol
CaZy	carbohydrate active enzymes
RT-PCR	real time polymerase chain reaction
CBM	Carbohydrate binding module
pNPC	*p*-nitrophenyl-β-D-cellobiase
pNPG	*p*-nitrophenyl-β-D-pyranoglucoside
MULac	p-nitrophenyumbelliferyl-β-D-lactoside
CD	circular dichroism
DNS	beta-glucosidase
EG2	endoglucanase 2 (Cel5A)
CBH1	cellobiohydrolase 1 (Cel7A)
GH(s)	glycosyl hydrolase(s)
CBP	consolidated bioprocessing
SSF	simultaneous saccharification and fermentation
SSCF	simultaneous saccharification and co-fermentation
HMF	5-hydroxymethyl furfural
SA	syringaldehyde
TAN	tannic acid

CON	coniferyl aldehyde
AC	acetic acid
FA	formic acid
FURF	furfural
4HB	4-hydroxymenzaldehyde
4HBA	4-hydroxybenzoic acid
ACET	acetophenone
VAN	vanillin
CIN	cinnamic acid
GRAS	generally regarded as safe
DSF	differential scanning fluorimeter
NMR	nuclear magnetic resonance
CD	circular dichroism

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Chapter 1

GENERAL INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 GENERAL INTRODUCTION

Concerns about the instability of fuel prices, the environmental impacts of fossil fuels and energy security have led to global interest in exploring energy alternatives that are sustainable, renewable and environmentally friendly (Mohanram et al. 2013, Kapu et al. 2013). Transition from a fossil fuel-based economy to a bio-based economy has the potential to address pressing challenges such as energy security and global climate change (Gao et al. 2014). Current distilleries operating in countries like the USA and Brazil are based on sugar or starch feedstocks, which are also used for human food and animal feeds (Hahn-Hägerdal et al. 2006). Use of these edible food and sources is controversial as applying them as feedstock for bioenergy production may pose a threat to food security (Sakai et al. 2007, Allen et al. 2010). Therefore, there is a compelling need for alternative energy sources and this is aggravated by the increasing demand for sustainable and reliable energy for transportation, heating and industrial processes, in particular those with minimum or no environmental impact (Hahn-Hägerdal et al. 2006). Lignocellulosic biomass provides an interesting alternative energy source and is the only foreseeable future for renewable transportation fuels (Kont et al. 2013, Klinke et al. 2004). Furthermore, the use of lignocellulosic biomass for biofuel production will create new markets and can thus revive the agricultural sector and improve rural development (Gao et al. 2014).

Lignocellulosic biomass is abundant in nature and its use as a substitute or partial replacement for fossil-derived fuels is gaining increasing attention (Anwar et al. 2014, Kapu et al. 2013). Approximately 1.3 billion mega-tons of lignocellulose biomass are produced annually across the globe (Goyal et al. 2011). However, the recalcitrant nature of the biomass material and other techno-economic problems are hindrances in developing commercial processes for converting lignocellulosic biomass into bioenergy (Gao et al. 2014). To fully harness the energy potential in lignocellulose biomass, barriers such as the recalcitrant nature of the plant biomass and the cost of the enzymatic hydrolysis process have to be addressed (Goyal et al. 2011). Lignocellulosic biomass recalcitrance is due to the association of sugar polymers with lignin, forming a tough structure that protects the plant from foreign invasion. Pretreatment of the plant material is therefore necessary to weaken this tough structure prior to hydrolysis and fermentation. One method that is gaining preference for industrial application is the steam pretreatment process (Palonen et al. 2004) during which lignin is structurally modified and hemicellulose is hydrolysed to monomeric

sugars to yield a cellulose-lignin fraction that is more amenable to enzymatic hydrolysis (Szengyel and Zacchi 2000).

However, during the pretreatment process and fractionation of lignocellulosic biomass, a wide spectrum of inhibitory by-products are also released, mostly from the hemicellulose fraction (Sakai et al. 2007). About 60-90% of the weight of the total solid fraction (filter cake) also absorbs the prehydrolysate liquid that contains inhibitory degradation compounds (Jing et al. 2009). For economic reasons, it is desirable that all sugars including those released from the hemicellulose fraction be converted to ethanol (Heer and Sauer 2008). This requires that enzymatic hydrolysis proceeds in the presence of a certain level of inhibitors, whether the hemicellulose fraction is removed from the filter cake or not (Klinke et al. 2004). These degradation products include organic acids (acetic, formic and levulinic acid), furan derivatives (furfural and 5-Hydroxymethyl furfural, HMF), phenolic compounds (including vanillin, syringaldehyde and others) and a variety of other small molecular weight compounds (Szengyel and Zacchi 2000; Jing et al. 2009). The composition and concentration of these degradation products vary depending on the type of lignocellulosic biomass, preteatment type and conditions - such as temperature, time, pressure, catalyst and redox conditions (Sakai et al. 2007; Jing et al. 2009). The trends of cellulase inhibition during enzymatic hydrolysis have not been clearly elucidated since most reports used complex commercial cellulase preparations and were based on different biomass substrates and different pretreatment methods (Jing et al. 2009).

Cellulases and hemicellulases have various applications in different industrial processes such as biological pulping, wastewater treatment, bleaching of chemical pulps, modification of waste paper and conversion of industrial and agricultural by-products to valuable commodities (Kaya et al. 2000). Industrial processes employing enzymes as catalysts are environmentally sound as enzymes reduce the use of hazardous chemicals and create a niche for exploring sustainable and renewable technologies. However, the efficiency of enzymes in industrial processes is highly dependent on variable factors such as pH, temperature, enzyme-substrate ratios, reaction time, agitation intensity and inhibitors that may limit their performance. Maximum performance of enzymes thus requires optimisation of the reaction and process control (Kaya et al. 2000). In lignocellulosic biomass conversion, optimum combinations of cellulases, hemicellulase and other complimentary enzymes are required for complete hydrolysis of lignocellulosic biomass (Van Dyk and Pletschke 2012). The cellulases required for the breakdown of cellulosic polymers include endoglucanases

that cleave cellulose amorphous regions and expose reducing and non-reducing chain ends for the action of cellobiohydrolases. Cellobiohydrolases then hydrolyse the exposed reducing and non-reducing chain ends of the crystalline cellulose and release cellobiose and cellooligossacharide units (Varnai et al. 2013), which in turn is cleaved to glucose by the action of β-glucosidases.

Over the past decade, enzyme companies have reduced the cost of enzyme production by 20-fold and the required enzyme loading has been remarkably reduced to between 2-10 mg/g lignocellulose solids under certain conditions (Varnai et al. 2013, Gao et al. 2014). However, enzymatic hydrolysis of lignocellulose hydrolysate is still one of the major expensive steps in biofuel processing as it represents approximately 15-20% of total ethanol production costs (Varnai et al. 2013). To achieve an economically feasible and attractive ethanol production process, further reduction in enzyme cost is critical and can be achieved for an example, through enzyme recycling strategies (Gao et al. 2014).

There are considerable research efforts on developing optimum enzyme mixtures in order to minimise the amount of enzymes required and make the process more economically feasible and attractive (Van Dyk and Pletschke 2012). The major concern with decreasing enzyme loadings is that it can result in increased phenol:protein ratios during the hydrolysis, which will amplify the inhibition effect by phenolic compounds (Ximenes et al. 2011). Further decreases in the cost of enzymatic hydrolysis and overall processing could be focused on first understanding the role played by lignin and associated residues in inhibiting cellulases and factors promoting enzyme loss (Gao et al. 2014). This is important as the high affinity of cellulases for ligneous surfaces prevents the free movement of enzymes, which is necessary for efficient degradation of insoluble lignocellulose sugar polymers (Rahikainen et al. 2011). More information on the inhibitory profiles of pretreatment by-products will accelerate the rational design of pretreatment technologies that are tailored to remove or chemically modify certain lignin components and will also guide in the engineering of robust cellulases and ultimate reduction of enzyme loading and hydrolysis costs (Jing et al. 2009, Gao et al. 2014).

1.2 AIMS AND OBJECTIVES

The aims of the study were to closely investigate the interaction between recombinant cellulases and inhibitory by-products that result from the pretreatment of lignocellulosic biomass that inhibit/deactivate enzymatic hydrolysis and fermentation steps during biomass

conversion to biofuels. Furthermore, detoxification strategies were explored as they hold promise to alleviate inhibition during both enzymatic hydrolysis and fermentation and have the potential to allow for simultaneous saccharification and fermentation (SSF) or consolidated bioprocessing (CBP). The specific objectives followed to achieve these aims were as follows:

(i) A systematic approach was followed to investigate the role of individual lignocellulose-associated inhibitor compounds in the inhibition and/or deactivation of the cellulases TeCel7A-TrCBM, TrCel5A and SfCel3A. The impact of selected inhibitor compounds on the hydrolysis of Avicel was also investigated using a combination of TeCel7A-TrCBM and TrCel5A with the addition of Novozyme 188 Cel3A to prevent feedback inhibition from cellobiose. Most studies have focused on commercial enzyme preparations with complex mixtures of cellulase and enhancers. The approach to use individual recombinant enzymes aimed to shed light on the impact of these inhibitors on each individual cellulase.

(ii) Exploring different sulfonating agents as enhancers of enzymatic hydrolysis and potential tools to alleviate inhibition in biomass conversion processes. Sulfur oxyanions (sodium dithionite and sodium sulfite) normally employed during the delignification process in the papermaking industries were selected as *in situ* detoxifying agents. In addition, the detoxification effect of reducing agents as well as enzymatic detoxification with laccase enzyme was studied using a selection of individual inhibitor compounds.

(iii) The mechanism(s) involved in the inhibition and/or deactivation of cellulases by inhibitor compounds was investigated on the major cellulase, Cel7A, and selected inhibitor compounds based on their inhibition/deactivation strength observed in objective (*i*). We also investigated the effect of CBM in the non-productive adsorption of Cel7A. The inhibition effect of selected inhibitory compounds on TeCel7A fused with the TrCBH1-CBM domain was investigated using a fluorescence-based technique, differential scanning fluorimetry (DSF). This technique involves real-time tracking of protein unfolding induced by temperature increase or ligand binding. We attempted to understand the role of the CBM domain fused to the carboxyl terminal domain of the TeCel7A on the stability of the protein in the presence of various inhibitor compounds.

The dissertation is presented as a compilation of six chapters. Chapter 2 comprises a literature review that covers the scope of literature relevant to the study topic. Chapters 3 to 5 are presented as research manuscripts that cover the aims of the study topic. Chapter 6 includes a general discussion and final conclusions. The first objective was addressed in Chapter 3 and was published as: "*Lignocellulosic hydrolysate inhibitors selectively inhibit/deactivate cellulase performance"* in *Enzyme and Microbial Technology* **81,** 16-22. The second objective was addressed in Chapter 4, where different chemical compounds and laccase enzyme were investigated for their potential as detoxifying agents in the enzymatic hydrolysis of steam pretreated sugarcane bagasse and pure crystalline cellulose. The third objective was addressed in Chapter 5 where the mechanism(s) of inhibition/deactivation and the role of CBM on the non-productive adsorption of cellobiohydrolase (TeCel7A) were investigated.

1.3 OUTCOMES

The outcomes of this work contributed to the broadening of the fundamental knowledge and understanding of inhibition and/or deactivation of cellulases by lignocellulose-associated inhibitor compounds. This study also expanded on the knowledge regarding potential strategies to alleviate inhibition and enhance enzymatic hydrolysis, as well as the potential of employing a recently developed technique, DSF, to trace the impact of cellulase-inhibitor binding on the stability of the protein.

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Chapter 2

LITERATURE REVIEW

INHIBITION OF CELLULASES BY LIGNOCELLULOSE BIOMASS PRETREATMENT ASSOCIATED BY-PRODUCTS

Inhibition of cellulases by lignocellulose biomass pretreatment associated by-products

2.1 INTRODUCTION

The increasing energy demands for transportation, domestic use and industrial processes are a huge global concern and alternative energy sources to fossil fuel are required to meet future energy demands (Sun and Cheng, 2002; Jonsson and Martin, 2016). Currently, increasing energy demand is largely accommodated by crude oil and this demand is projected to increase up to 116 million barrels by 2030. Increasing dependence on fossil-derived fuels leads to increasing damages to the environment due to high emissions of greenhouse gases from fossil fuels (Hahn-Hägerdal et al., 2006; Allen et al., 2010). The instability in oil prices over the past few years is another driving factor that influences both public and scientific interest to obtain a sustainable alternative energy source that can reduce the heavy reliance on fossil fuels and improve energy security (Mohanram et al., 2013). An estimated 19% of energy consumed in the world in 2011 was from renewable energy resources such as wind, solar, geothermal, and hydrothermal and biomass. Biomass contributed about 13% of the total renewable energy bundle and was harnessed either by burning carbon-rich biomass or through thermochemical conversion of biomass into heat and power (Balan et al., 2014).

The use of alternative carbon sources (such as biomass), if done correctly, could generate energy, combat global warming and improve energy security in future (Sakai et al. 2007). Biofuels can be produced in different forms, including liquid, solid and gaseous states, from biological material. First generation biofuels uses sugarcane or grains as raw material, whereas second generation is based on lignocellulosic biomass (Mohanram et al. 2013). Annually, approximately $1{,}3\times10^{10}$ MT lignocellulose biomass is produced globally (Den Haan et al. 2007b, Goyal et al. 2011). This makes lignocellulosic biomass the only foreseeable, feasible and sustainable energy feedstock (Lynd et al. 1999, van Zyl et al. 2011). Lignocellulosic biomass is abundant as agricultural and forest residues, industrial and municipal wastes and special crops dedicated for energy production (Klinke et al. 2002; Yang et al. 2011, Jönsson et al. 2013) and can contain up to 70% carbohydrate as cellulose and hemicelluloses (Klinke et al. 2004) and up to 30% lignin (Picart et al. 2014).

Almost all large-scale ethanol production, for example in the USA, Canada and Brazil, relies on edible parts of plants, mostly sugarcane juice and corn starch (Sakai et al. 2007, Hahn-Hägerdal et al. 2006). In the USA, ethanol from cornstarch is used as a partial replacement for gasoline containing up to 10% volumetric ethanol. However, these first generation biofuels directly compete for arable land with crops dedicated for the production of human food and animal feeds and rely on technologies that are more expensive than existing fossil fuel conversion technologies (Sun and Cheng 2002). Biofuel feedstocks that are also essential food sources will therefore not be sufficient to supply global energy demands (Allen et al. 2010). In addition, the total greenhouse emissions from first generation biofuels frequently approach that of fossil fuels (Mohanram et al. 2013).

Depending partly on the future prices of biomass technologies, lignocellulose biomass-derived biofuels are likely to become part of the solution to the challenge of shifting liquid fuels in the transport sector towards more sustainable energy sources than petroleum products (Sims et al. 2010; Lynd et al. 2015). Production of biofuels from lignocellulosic biomass could significantly contribute to improving energy security, reduction of dependence on (imported) oil, reduction of greenhouse gas emissions, support for agricultural industries and economic development, especially in rural or farming areas (Sun and Cheng 2002, Allen et al. 2010). There are increasing initiatives involved in the development of technologies for the conversion of lignocellulose biomass to biofuels. The success of these initiatives will depend on governmental subsidies to render the process economical with cellulosic ethanol prices that are comparable to that of gasoline fuel (Varnai et al 2013; Balan et al. 2013).

The transport sector is the major consumer of liquid fuels such as petroleum (Mohanram et al. 2013). With the growth in the advanced biofuel landscape (cellulosic ethanol, biomass-based diesel, biobutanol, bio-oil, green gasoline and jet-fuel) proposed to take off in the near future, it is estimated that biofuels will be able to meet more than a quarter of the world's transportation oil demands by 2050 (Mohanram et al. 2013). In the USA, a target of 36 million gallons of advanced biofuels produced per year was set for 2022. Currently, there are nine full demonstration plants and six commercial scale plants in operation in the USA. Most industrial projects in the USA have adopted biochemical conversion routes (e.g. Amyris, Abengoa Bioenergy and Butamax^{TM}) for the production of biofuels, whereas others adopted thermochemical (e.g. British Airways and Solena) or hybrid (e.g Swedish Biofuels and Lanza Tech) routes. These projects also include other advanced biofuels such as liquid

hydrocarbons (Amyris) and biobutanol (Butamax, Cobalt and Gevo) in addition to bioethanol (Dina et al. 2012; Balan et al. 2013).

In the European Union (EU), a 20-20-20 target was set in 2007 that aims to meet a 20% increase in renewable energy share and also improve energy efficiency by 20% in 2020 (Balan et al. 2013). In the EU, a balance between biochemical and thermochemical routes has been adopted as opposed to a preference for biochemical routes in the USA. Abengoa, Biogasol, Dong Inbicon, Chemtex are examples of industrial scale-up initiatives in the EU. The technologies initiated in the USA and the EU are likely to expand to other regions as first instalments, replicon, or extension of projects already initiated. As evidence, Abengoa, M and G/Chemitex, Swedish Biofuels and British airways/Solena are already expanding beyond these regions, which is a good indication that these industrial development initiatives based on second-generation biofuels will also have global impacts (Balan et al. 2013). In South Africa, a Biofuel Industrial Strategy adopted in 2007 included lower mandated substitution targets of 2 percent for liquid biofuels by 2013. However, the main objectives of this biofuel strategy was not to decrease heavily reliance on fossil derived fuels but to address socio-economic problems facing the rural communities and these included the alleviation of poverty, reviving the agricultural sector and creating employment opportunities (Kohler 2016)

Although there are positive developments in initiatives aimed at producing biofuels, there are still a number of techno-economic challenges that need to be overcome (van Zyl et al. 2007; Varnai et al. 2011). The technologies employed for the conversion of lignocellulose biomass (Fig. 2.1) to ethanol are currently inefficient and costly. More economically competitive pretreatment strategies that address the recalcitrance of biomass are being researched and evaluated at pilot and demonstration scale (Balan et al. 2013). The other major bottleneck is in the enzymatic hydrolysis step where enzyme production and enzyme dosages required to achieve efficient conversions, represent a barrier in commercialising lignocellulosic ethanol. A major area of concern during the enzymatic hydrolysis is the non-productive adsorption of enzymes to lignin residues. It is therefore important to understand the contribution of the enzyme adsorption-desorption mechanism on the loss of enzymes during the hydrolysis of lignocellulosic biomass, as well as the effects of this phenomenon on enzyme loading requirements and the recyclability of the enzymes (Varnai et al. 2011;Allen et al. 2010;van Zyl et al. 2007).

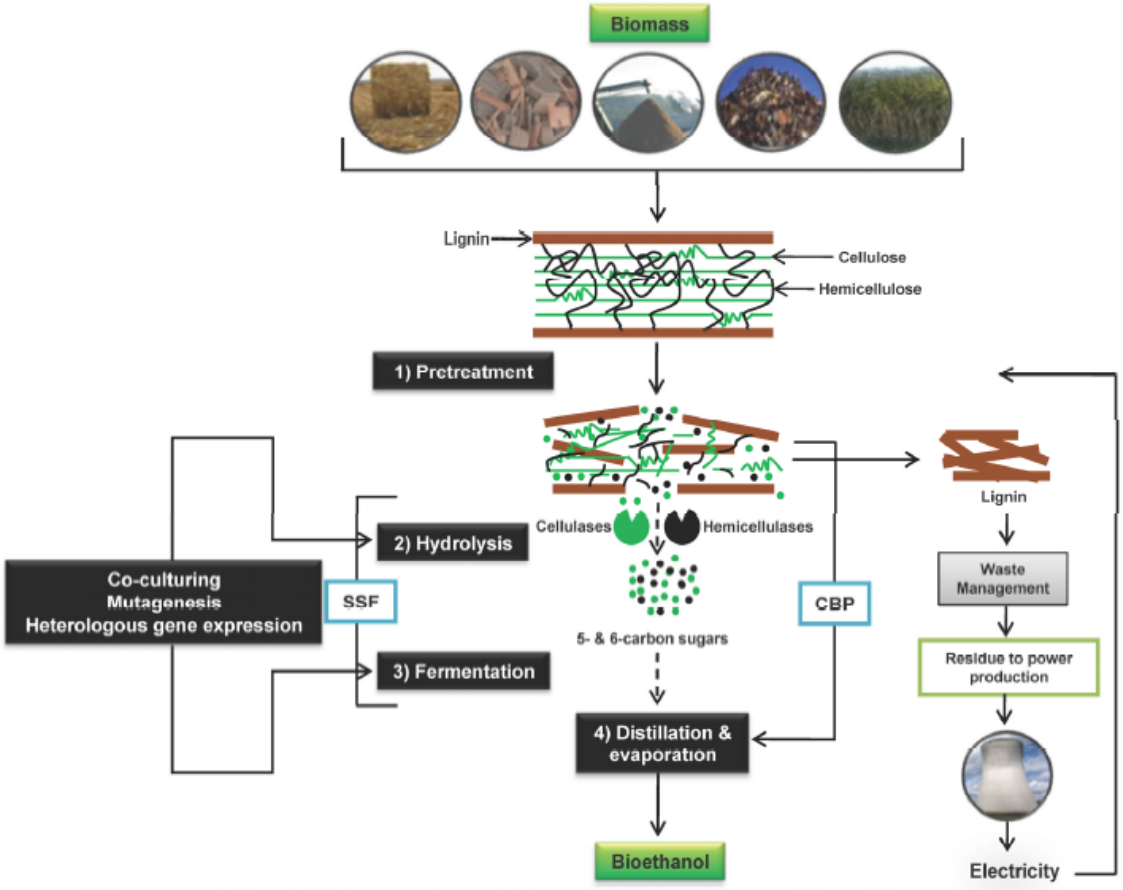

Fig. 2.1: Schematic representation for the conversion of lignocellulose biomass to ethanol Source: (Dashtban et al. 2009). Simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP)

2.2 LIGNOCELLULOSE STRUCTURE

Plant material can be taxonomically divided into softwood (gymnosperms), hardwood (woody angiosperms) and annual plants (herbaceous angiosperms) such as grasses (Klinke et al. 2004). Plants are mainly composed of three major polymers, namely cellulose (40-60%), hemicellulose (20-40%) and lignin (10-25%) (Fig. 2.2) (Sun and Cheng 2002, Alriksson et al. 2011). However, lignocellulosic materials vary in composition depending on the source of the feedstock, which can also differ mainly due to genetic variability (Anwar et al. 2014).

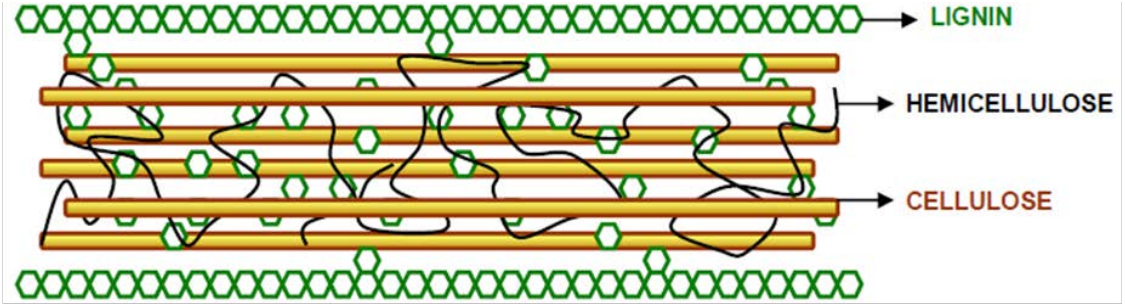

Fig. 2.2: Schematic representation of lignocellulose showing cellulose, hemicellulose and lignin components (Mussato and Teixeira 2010).

2.2.1 CELLULOSE

Cellulose is the principal homopolymer in lignocellulose, containing linear glucose units of up to 12 000 residues attached through *β*-1,4-glycosidic linkages (Mohanram et al. 2013; van Dyk and Pletschke 2012). The average molecular weight of cellulose is around 100 000 kDa. The glucan chains in cellulose are packed in a parallel orientation with the hydroxyl groups of glucose molecules forming strong intramolecular and intermolecular hydrogen bonding that result into a crystalline microfibril structure (Palmqvist and Hahn-Hagerdal 2000b, Mohanram et al. 2013, Varnai et al. 2014). Cellulose exists as aggregated bundles in the form of microfibrils that contain crystalline and amorphous regions. This structural organisation of cellulose increases its rigidity, contributes to its strong resistance to organic solvents and provides mechanical strength in plant cell walls.(Van Dyk and Pletschke 2012, Jung et al. 2012, Palmqvist and Hahn-Hagerdal 2000b). The cellulose microfibril spaces are filled with lignin and hemicelluloses in both the primary and secondary cell wall and middle lamellae, which shield cellulose from degradation. Only the amorphous parts of cellulose are easily degradable (Van Dyk and Pletschke 2012).

2.2.2 HEMICELLULOSE

Hemicellulose is a highly branched heteropolymer with its sugar composition, types of linkages, branching and substitutions varying between plant species. It is typically composed of pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and uronic acids (Mohanram et al. 2013, Kont et al. 2013). Hemicellulose is the second most abundant sugar polymer in plant cell walls and contributes to the heterogeneity of the plant (Jung et al. 2012). The average molecular weight of hemicellulose is >30 000 kDa. In different hemicelluloses, xylans and mannans are the most common sugar backbones with

other trace polysaccharides such as galactans, arabinans and arabinogalactans (Anwar et al. 2014).

In hardwood and annual plants, xylan is the major hemicellulose polymer. Xylan forms the hemicellulose backbone with xylopyranosyl residues linked by *β*-1,4-glycosidic bonds, which are mainly substituted with arabinose or glucuronic acid at their 2-O and/or 3-O positions (Mohanram et al. 2013). Xylan forms hydrogen bonds with cellulose and is covalently linked to lignin (Mohanram et al. 2013, Kont et al. 2013). Hardwood xylan is also characterised by high levels of acetylation (Palmqvist and Hahn-Hagerdal 2000b). Glucomannan is mainly found in softwoods and is composed of β-1,4-linked mannan and glucose residues, which are sometimes substituted by α-galactose. Other hemicelluloses include xyloglucan that consist of β-1,4-linked glucose residues with α-linked xylopyranose residues substituting more than half of β-1,4-linked glucose, and also mixed-linkage β-glucans consisting of β-1,3- and β-1,4-linked glucose residues. Hemicellulose forms a complex network with cellulose fibrils and these complexes in turn are embedded in the lignin matrix (Van Dyk and Pletschke 2012, Mohanram et al. 2013, Kont et al. 2013).

2.2.3 LIGNIN

Lignin is a poly-condensate of dehydrogenate products from the lignin precursors, *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Fig. 2.3), which forms a protective seal or glue that fills the spaces around cellulose and hemicellulose (Klinke et al. 2004, Hasunuma and Kondo 2012). The aromatic rings in these monomers are normally referred to as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), respectively (Klinke et al. 2004). Lignin polymer contributes 20 to 30% in plant biomass and is cross-linked or forms physical complexes with the polysaccharides, cellulose and hemicelluloses to provide the plant structure the necessary rigidity and recalcitrance (Larsson et al. 2000, Tejirian and Xu 2011). Lignin serves as a protective barrier that provides the plant with strength and protection against pathogens (Van Dyk and Pletschke 2012). The ratio of the H/G/S monomers varies depending on the plant species: in softwood lignin, the G-type monomers dominate with small proportions of H-type monomers, while the G-type and S-type monomer are present roughly in equal amounts and contains small quantities of H-type monomers in hardwood lignin. In addition to H/G/S monomers, the herbaceous plants also contain *p*-hydroxycinnamic acids (*p*-coumaric acid, ferulic acid and sinapic acids) in their lignin (Klinke et al. 2004).

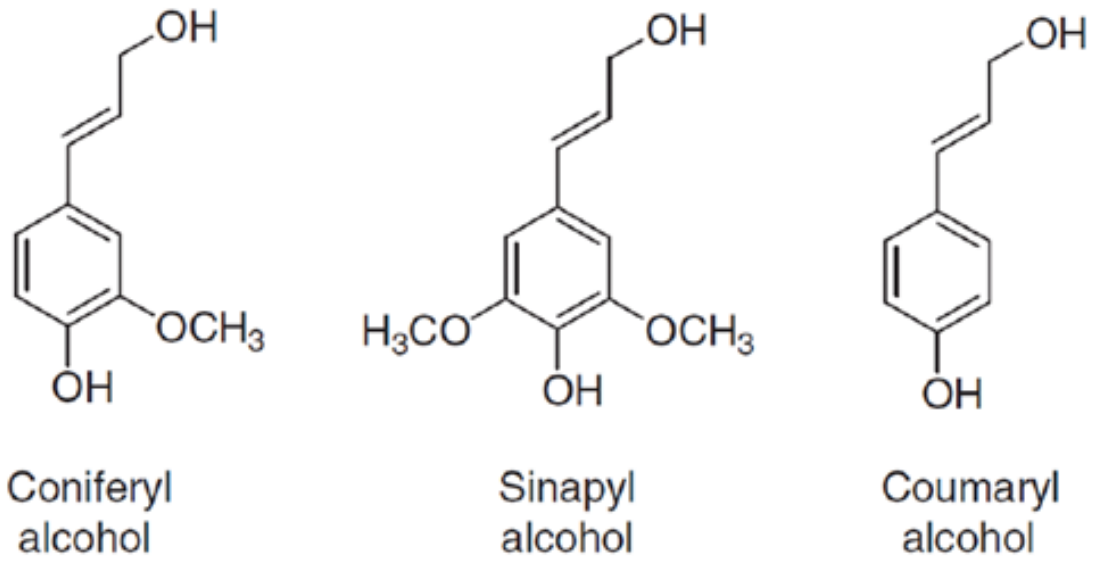

Fig. 2.3: Lignin precursors making up lignin polymer (Dina et al. 2012)

2.3 PRETREATMENT AND FORMATION OF DEGRADATION PRODUCTS

A number of challenges hinder the efficient and cost-effective conversion of lignocellulosic biomass to ethanol (Lewis Liu et al. 2008). The recalcitrance of the plant material and the cost of enzymes required for depolymerisation of the polysaccharides in plant biomass contribute a significant cost in the production of biofuels (Hendriks and Zeeman 2009, Kumar and Wyman 2009). Pretreated lignocellulose is fractionated into a soluble hemicellulose fraction and an insoluble filter cake fraction. The hemicellulose fraction contains mainly hemicellulose-derived monomeric sugars and degradation compounds, whilst the solid filter cake fraction is composed mainly of cellulose and insolubilized lignin (Lewis Liu et al. 2008, Jing et al. 2009). However, a considerable amount of the hemicellulose fraction also adsorb to the filter cake fraction after pressing. The filter cake is normally washed several times to remove the adsorbed soluble hemicellulose fraction leaving the water insoluble solid (WIS) fraction (Jing et al. 2009). The lignin residues and degradation compounds in the absorbed hemicellulose fraction significantly affect the efficiency of enzymatic hydrolysis in the subsequent enzymatic hydrolysis step by limiting cellulase access to substrate and inhibiting and/or deactivating cellulases (Ximenes et al. 2011, Zheng et al. 2013).

There are a number of pretreatment strategies that can be used to fractionate lignocellulose biomass and these strategies have been reviewed elsewhere (Sun and Cheng 2002, Alvira et al. 2010). Physico-chemical methods are often applied to disrupt lignin in order to gain access to the shielded polysaccharides. During the pretreatment process, lignin can be

modified by demethylation and partially solubilised to simple and oligomeric phenolics (Larsson et al. 2000). The most widely applied pretreatment processes in commercial applications are hydrothermal pretreatments which includes dilute acid catalysed pretreatment, liquid hot water pretreatment and steam explosion pretreatment. These pretreatment strategies proceed at varying conditions but the chemical and physical alterations in the structure of the pretreated material using these strategies are similar. These pretreatment strategies remove the water-soluble hemicellulose fraction while leaving the solid filter cake fraction that is mainly composed of cellulose and insoluble lignin (Jönsson et al. 2013, Ko et al. 2015a). It is preferable that all sugars present in hemicellulose and cellulose be converted to ethanol to increase the yield and improve process economics. The solid filter cake fraction also contains non-sugar compounds that are inhibitory to enzymatic hydrolysis, microbial growth and ethanol production (Heer and Sauer 2008). Enzymatic hydrolysis is therefore expected to be associated with a certain level of inhibitors (Zheng et al. 2013).

The nature and concentration of the degradation products released during pretreatment of lignocellulose biomass depend on a number of pretreatment parameters, including temperature, time, pH, pressure, redox conditions and catalytic compounds that were added (Klinke et al. 2004). The by-products released during pretreatment can be divided into sugar degradation products (furans and organic/weak acids) and various lignin-derived phenolic compounds (Fig. 2.4) (Heer and Sauer 2008, Jung et al. 2012).

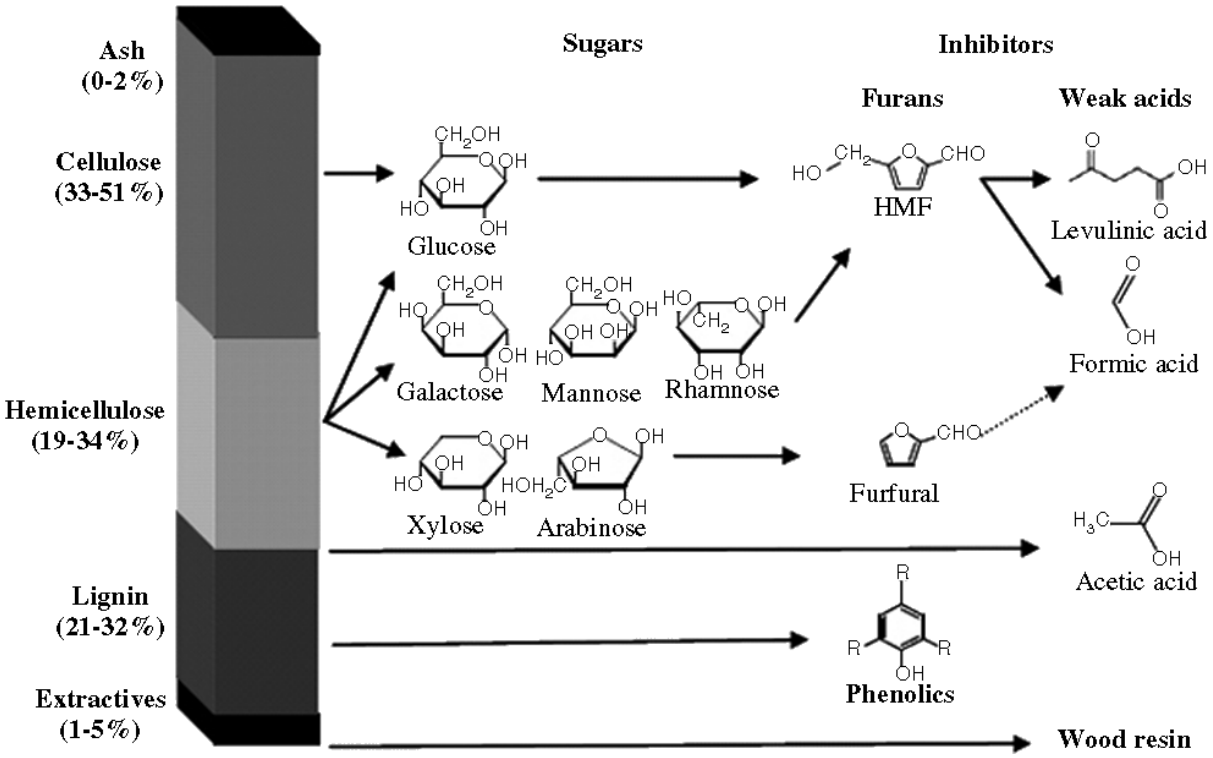

Fig. 2.4: Pretreated lignocellulose biomass and its hydrolysate products (Almeida et al. 2007).

2.3.1 SUGAR DEGRADATION PRODUCTS

Pretreatment under acidic conditions and high temperatures leads to pentose and hexose sugar degradation and to the formation of furan aldehydes, such as furfural and 5-hydroxymethyl furfural (HMF), respectively. These compounds inhibit enzymatic hydrolysis, growth of a fermenting microorganisms and prolong the lag phase of growth (Almeida et al. 2007). Depending of the pretreatment conditions, the furan aldehydes can be further degraded to formic acid and levilinic acid. Hemicellulose in plant biomass is typically acetylated and the acetyl groups are released as acetic acid under acidic conditions (Klinke et al. 2004). Various aromatic compounds are also present in hydrolysates and their concentrations content depends on the pretreatment type and conditions as well as the H/G/S ratio of the lignin in biomass (Klinke et al. 2004).

Organic acids (acetic, formic and levulinic acid) are amongst the most toxic compounds present in lignocellulose hydrolysate (Larsson et al. 1999a; Klinke et al. 2004). One particular compound of interest (due to its high commercial value) is acetic acid, which is found in high quantities in hardwoods which is extensively acetylated compared to

softwoods. Acetic acid is typically found in higher concentrations (1 to 10 g/L) than formic acid in lignocellulose hydrolysates (Almeida et al. 2007, Du et al. 2010, Jing et al. 2009), but formic acid toxicity effects are more pronounced than that of acetic acid (Hasunuma and Kondo 2012, Larsson et al. 1999). Increasing concentrations of organic acids (acetic, levulinic and formic acid) also decrease ethanol yield and volumetric productivity, whereas lower concentrations of these organic acids can favour the production of ethanol (Jing et al. 2009, Almeida et al. 2007).

2.3.2 LIGNIN DERIVED PHENOLIC COMPOUNDS

Phenolic compounds are liberated from the partial hydrolysis of lignin and vary in content and concentration depending on biomass type, pretreatment conditions and biomass loading (Almeida et al. 2007). Increasing the biomass solid content and reducing liquid volumes also result in increased soluble phenolic concentration. Other phenolic compounds in the lignocellulose hydrolysate originate from extractives and possibly from the degradation of sugars. Phenolic compounds in higher plants can be divided into low molecular weight monomeric and polymeric compounds. Phenols in higher plants primarily play a role in plant defence mechanism against plant pathogens, which also prevents plant degradation and hydrolysis by enzymes generally secreted by plant pathogens (Ximenes et al. 2011).

The most common phenolic compounds found in hydrolysates are 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, vanillin, dihydroconiferyl alcohol, coniferyl aldehyde, syringaldehyde and syringic acid (Kaya et al. 2000; Klinke et al. 2002, 2004). Other phenolic compounds such as the derivatives of cinnamic acids (ferulic acid and *p*-coumaric acid) are produced during the hydrolysis of esterified hemicellulose and lignin from soda pulping or wet oxidation of wheat straw. Furthermore, oxidative cleavage of conjugated double bonds can also form 4-hydroxybenzoic acid and vanillic acid. Phenols found in hydrolysates can be divided into H/G/S types based on their degree of methylation and also based on their functional groups as aldehyde, ketones, acids, alcohols, etc. (Klinke et al. 2004, Almeida et al. 2007). Other low molecular weight aromatic compounds form from wood extractives as a result of side reactions mostly during heating in acid-catalyzed pretreatment (Larsson et al. 2000). These extractives can include terpenes, phenols, quinines and tannins and non-extractives including pectins, protein and ash components. The 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid are also suggested to be derived from extractives rather than from lignin. The extractives play a role in the chemotaxonomic division and anti-microbial activities of plant species (Klinke et al. 2004).

2.4 GLYCOSIDE HYDROLASES

Glycoside hydrolases (GHs, EC 3.2.1) represent a broad group of enzymes that catalyse the cleavage of glycosidic bonds in carbohydrates (Zhang et al. 2013). GHs are widely distributed into 133 families in the Carbohydrate Active enZYmes, or CaZy (www.cazy.org) database based on sequence similarities and predicted structures (Lombard et al. 2014). The primary function of GHs is to cleave glycosidic bonds in glycosides, glycans and glycol-conjugates. The natural substrate for GHs varies from di-, oligo-, polysaccharides and polysaccharide derivatives (Naumoff 2011).

2.4.1 ENZYME PRODUCTION AND COST

Many fungal species possess a lignocellulolytic enzyme system and they are among the most efficient cellulose degraders known to man (Fujita et al. 2002, Fujita et al. 2004). The fungus, *Trichoderma reesei*, is the best studied Ascomycete and carries 28 glycoside hydrolase (GH) genes encoding enzymes required for the degradation of plant biomass. The *T. reesei* hydrolases are further categorised into seven cellulolytic, 16 hemicellulolytic and five pectinolytic enzymes, with the major secreted GHs being cellobiohydrolases and endoglucanases (Fujita et al. 2002, Olsen et al. 2011, Varnai et al. 2014). The total protein secreted by *T. reesei* cultured on a cellulosic substrate consists of approximately 60% cellobiohydrolases (TrCel7A and TrCel6A), 20% endoglucanases (TrCel7B, TrCel5A and TrCel12A) and 12% β-glucosidase (TrCel3A) (Teugjas and Valjamae 2013; den Haan et al. 2013, Kont et al. 2013).

Glycoside hydrolases account for a significant share of industrial enzymes available in the market and their market is rapidly growing (Zhang and Zhang 2013). Commercial enzymes available in the market are produced by companies like Novozyme, Genencor, Iogen, etc. from *Trichoderma* and *Aspergillus* (Zhang and Zhang 2013). There is an increasing demand for cellulases for various industrial applications in textile industry, pulp and paper industry, food industry, additives, in detergents and for improving the digestibility of animal feed. GHs play a key role in the development of biofuels. In biofuels, they are important during the enzymatic conversion of biomass to fermentable sugars, which are subsequently converted to bioethanol (Vuong and Wilson 2010, Zhang and Zhang 2013). A typical cellulose production plant uses submerged fed-batch fermentation to produce low-cost cellulases.

An ever-increasing body of research has focused on the improvement of the catalytic efficiency of cellulases by different engineering strategies (Zhang and Zhang 2013). Over the decades, enzyme companies have reduced the cost of enzyme production by 20-30 fold through improving (i) thermostability, (ii) specific activity in enzyme cocktails and (iii) reduction of sugar costs from lactose and glucose. However, enzymatic hydrolysis still represents approximately 15% of total ethanol production costs thus making enzymes the third highest cost component after capital and feedstock costs (Stephen et al. 2012; Varnai et al. 2013). To achieve an economically feasible and attractive ethanol production process, further reduction in enzyme cost and the cost of hydrolysis is critical (Zhang and Zhang 2013, Varnai et al. 2013, Pakarinen et al. 2014). Other strategies include reducing end-product inhibition, better synergistic cooperation of enzyme mixtures (Van Dyk and Pletschke 2012), efficient production processes as well as enzyme recycling strategies (Pakarinen et al. 2014, Pribowo et al. 2012). However, these attempts have not resulted in drastic cellulase activity enhancement. It is clear that a better understanding of enzymatic hydrolysis mechanisms as well as the relationship of cellulases with other biological elements in the reactor environment is necessary to engineer enzymes with robustness, high catalytic activity and specificity (Zhang and Zhang 2013).

2.4.2 DEPOLYMERISATION OF LIGNOCELLULOSIC BIOMASS

Complete hydrolysis of cellulose requires a combination of three main types of cellulases: (i) endoglucanase (mainly Cel5A, EC 3.2.1.4), (ii) exoglucanases such as cellobiohydrolases (Cel7A, EC 3.2.1.176 and Cel6A, EC 3.2.1.91), and (iii) β-glucosidase (Cel3A; EC 3.2.1.21) (Hasunuma and Kondo 2012). Endoglucanases randomly cleave the amorphous region in cellulose chains to produce reducing and non-reducing ends for the action of cellobiohydrolases. The cellobiohydrolases cleave the crystalline regions in cellulose; Cel7A hydrolyses act processively from the reducing ends while Cel6A hydrolyses from the non-reducing ends, resulting in the release of short oligosaccharides and cellobiose (Hall et al. 2011, Fujita et al. 2002, Fujita et al. 2004). Cellobiose is subsequently hydrolysed by cellobiases (β-glucosidases) that bind non-reducing glucose units in cellobiose and cleave them off to produce glucose (Zhang and Zhang 2013) (Fig. 2.5).

Recent findings has described the role of lytic polysaccharide monooxygenases (LPMOs) in introducing chain ends for cellobiose in cellulose degradation. These novel oxidative enzymes randomly cleave cellulose by an oxidation reaction in one of the two new chain ends resulting from endoglucanase action. This introduces a C1 or C4 oxidation at the

released reducing or non-reducing chain ends, respectively (Horn et al. 2012, Varnai et al. 2013). Synergistic interaction of multiple enzymes in the depolymerisation of cellulosic substrates is necessary to achieve high product yields. In addition to cellulases, other complimentary and accessory enzymes such as hemicellulases are required to reduce steric hindrance, thus further increasing the accessibility of cellulose to enzymatic hydrolysis (Varnai et al. 2014).

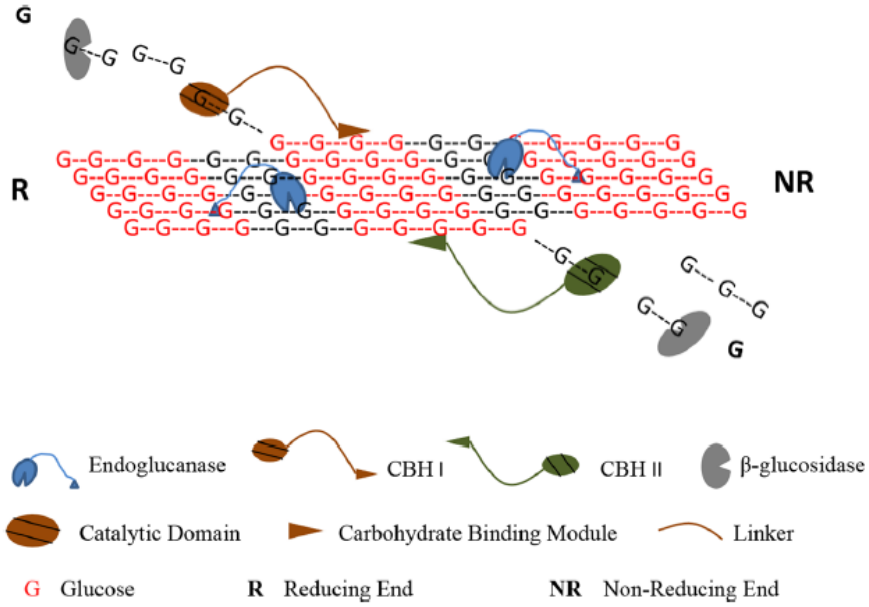

Fig. 2.5: Action of various cellulases on the surface layer of cellulose (Kumar and Murthy 2013). The action of enzymes from three main classes on cellulose is illustrated. Glucose molecules in red represents crystalline regions and glucose molecules in black represent amorphous regions.

Hydrolysis of lignocellulosic biomass is characterised by two distinct phases, a quick initial adsorption and a slow desorption of enzyme components, which can be described using the Langmuir isotherm (Varnai et al. 2014). Productive adsorption of cellulases to cellulose fibrils occurs rapidly, brings the enzyme in close contact with the insoluble substrate, and enables an efficient catalytic process (Haven and Jorgensen 2013). The hydrolytic action of cellobiohydrolases has been shown through microscopic studies to be processive and unidirectional on cellulosic fibrils. The processive nature of cellulases maintains the close association of the enzyme and its crystalline substrate to improve catalytic efficiency. The advantage of the processive strategy is that detached single chains are prevented from re-associating with the crystalline cellulose, but this also causes slow catalytic speed (Varnai et al. 2011; 2014). The lignin obstacles in the hydrolysis path-length limit the processivity of cellobiohydrolases and this requires enzymes with faster desorption abilities that cannot be

easily entrapped by lignin obstacles (Varnai et al. 2014). Ideally, the cellulase should desorb as the cellulose and hemicellulose is hydrolysed; however, processive cellulases remain adsorbed to cellulose chains (Haven and Jorgensen 2013).

2.4.3 STRUCTURAL PROPERTIES OF FUNGAL CELLULASES

Saprophytic fungi produce various extracellular cellulases that contain single or multiple domains (Varnai et al. 2014). The fungal cellulases are typically bimodular structures with the catalytic domain (CD) connected through a glycosylated peptide linker to the carbohydrate-binding module (CBM). The cellobiohydrolases also contains N- and O-glycosylations (den Haan et al. 2013, Pakarinen et al. 2014, Le Costaouec et al. 2013). With the exception of Cel12A, all *T. reesei* cellulases have a two-domain structure (Palonen et al. 2004). The *T. reesei* cellulases, Cel7A, Cel7B, Cel6A and Cel5A, have been extensively studied, with TrCel7A being the most investigated cellulase since it is produced in large quantities (Varnai et al. 2014). The Cel7A enzyme is composed of a 434 amino acid CD linked via a flexible and heavily O-glycosylated linker of 24 amino acids to a 36 amino acid CBM, which will be discussed in detail later in this chapter. The function of the linker is to separate these domains and transfer the energy required for processive motion from the catalytic domain (Hall et al. 2011).

2.4.3.1 Catalytic domain (CD)

Glycoside hydrolases (GHs) differ in the shape of their catalytic sites (Zhang et al. 2013). The catalytic site of cellobiohydrolases contains tryptophan residues in the entrance of their tunnel and in the inner lining, which is suggested to play a role in guiding the glucan chains into the catalytic site for processive catalysis. Cellulases can also bind through the catalytic site, but this phenomenon is less intensively studied. More studies have focused on cellulases carrying a CBM domain and considered CBM as a requirement for full functionality of cellulases on crystalline cellulose. The CD has comparable processivity speed and has the ability to load cellulose chains in its catalytic site (Varnai et al. 2014). The GHs have varied topologies that range from all β-sheet proteins to β/α-barrels to all α-helical proteins (Zhang et al. 2013). The topologies of enzyme active sites generally fall into three categories regardless of their family classification or whether they are inverting or retaining. These topologies include (i) cleft or groove, (ii) tunnel and (iii) a pocket-like active site (Davies and Henrissat 1995).

(i) Cleft/groove catalytic site

The cleft or groove-shaped active site is an open structure commonly found in endo-acting enzymes such as endoglucanases and xylanases (Davies and Henrissat 1995). This catalytic site allows for random binding of the enzyme to polymeric substrates (Davies and Henrissat 1995). The endoglucanases are non-processive cellulases that randomly bind and nick the cellulose chains to generate glucose and soluble cellodextrins. The fungal endoglucanases can occur as a single domain (only CD) or as a bimodular domain (CD with CBM attached through a linker peptide). Some endoglucanases are found to have other domains with unknown functions (Zhang and Zhang 2013, Varnai et al. 2014).

(ii) Tunnel-shaped catalytic site

The tunnel-shaped catalytic site is suggested to have evolved from the open structure or cleft-shaped active site where the protein evolved long loops that cover parts of the open catalytic site (Davies and Henrissat 1995). The polysaccharide chains are threaded through the tunnel and the products are released while the enzyme remains firmly attached to the polysaccharide chain. This creates a processive condition and this type of catalytic site has only been identified on cellobiohydrolases. The processivity is suggested to be a key factor for efficient enzymatic degradation of insoluble cellulose (Davies and Henrissat 1995). The Cel7A and Cel6A have a tunnel-shaped catalytic site formed by disulphide bridges where β-glycosidic bonds are threaded sequentially in a ‘processive’ manner. The disaccharides are cleaved through retaining (Cel7A) and inverting (Cel6A) mechanisms (Varnai et al. 2014, den Haan et al. 2013). The Cel7A is more processive than Cel6A, which has a shorter tunnel that might lead to earlier detachment from the substrate and re-initiation of new cleavage sites (Varnai et al. 2014). The TrCel7A amino acid sequence is 66% homologous to that of the cellobiohydrolase of *Talaromyces emersonii* (TeCel7A). Also their catalytic tunnels are structurally similar and span approximately 50 Å with 10 glycosylation sites (den Haan et al. 2013).

In addition, cellobiohydrolases from these two organisms consist of two β-sheets arranged to form a β-sandwich with long loops enclosing their catalytic tunnel (den Haan et al. 2013). The TeCel7A tunnel differs slightly from that found in TrCel7A since it is more open and straight to allow shorter oligosaccharide chains to the active site. The TrCel6A tunnel is formed through a barrel of α/β folds with seven parallel β-sheets and a roof enclosed by two loops. It is a short tunnel and can only accommodate four glycosyl units at the non-reducing end of cellulose chains. The cellobiohydrolases are stabilised by

interactions such as disulphide bond formation. In *T. reesei*, ten disulphide bonds are found in the CD and two in the CBM, whereas the single modular TeCel7A has only nine disulphide bonds (den Haan et al. 2013). The directionality of the enzyme motion along the chain may change depending on the enzyme mechanism (inverting or retaining) and the position of the cleavage point. TrCel7A proceeds towards the non-reducing ends while TrCel6A proceeds towards the reducing ends (Davies and Henrissat 1995).

(iii) Pocket-like shaped catalytic site

The pocket-like active site is typical in enzymes such as β-glucosidases and β-galactosidases and the topology allows for optimal recognition of non-reducing chain ends (Davies and Henrissat 1995). Beta-glucosidases (Cel3A) bind to non-reducing glucose units through its pocket shaped catalytic site and cleave glucose off from cellobiose or dextrin. Family 1 and 3 Cel3A use the retaining type mechanism and family 9 the inverting type (Zhang and Zhang 2013).

2.4.3.2 Carbohydrate binding module (CBM)

The carbohydrate binding modules (CBMs) are categorised into 66 families and only members of the family 1, 6 and 63 CBMs have cellulose binding affinity (den Haan et al. 2013). The *T. reesei* CBMs are small wedge-shaped folds with a cellulose-binding surface and three exposed amino acid residues required for binding to solid surfaces such as cellulose. The family 1 CBMs are the most studied and their planar face consists of conserved amino acid moieties that target the crystalline cellulose fibrils (Fig. 2.6). The CBMs in this family are attached to the C-terminus in Cel7A or to the N-terminus in Cel6A (Varnai et al. 2014, den Haan et al. 2013). Family 1 CBMs in TrCel7A has aromatic tyrosine residues Y31, Y32 and Y5, which together with polar side chains Q7, Q34 and N29, has been shown to be critical for CBM-cellulose binding (Rahikainen et al. 2013a).

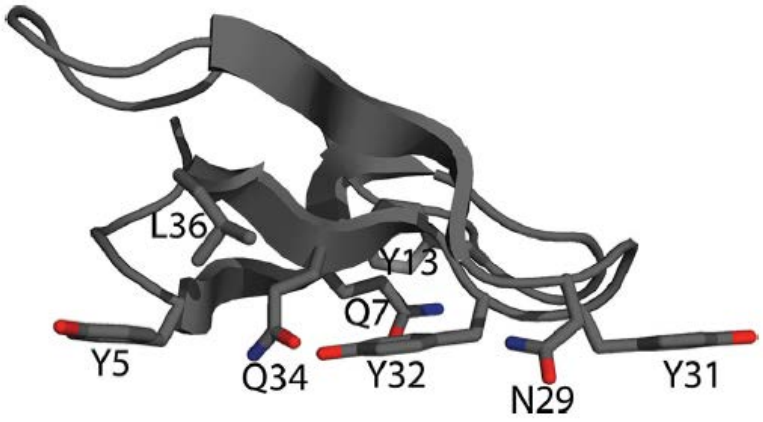

Fig. 2.6: The *T. reesei* Cel7A CBM structure displaying amino acid residues important for enzyme binding (Kathryn et al. 2015).

Simulation studies indicate that the four conserved residues exposed on the planar hydrophobic surface of CBMs, Y5, Q7, N29 and Y32 aid in timeous diffusion of the CBM across the cellulose surface (Rahikainen et al. 2013a; Kathryn et al. 2015). They further facilitate binding of CBM to cellulose through hydrophobic interactions between the aromatic acid residues on the face of the CBM and the pyranose rings of cellulose (Varnai et al. 2014, Rahikainen et al. 2013a). The amino acids are spaced to coincide with every second glucose ring on the glucan chains (Palonen et al. 2004, Strobel et al. 2015). This positioning of aromatic amino acid residues in the CBM is suggested to allow for the formation of van der Waals forces and aromatic ring polarization interactions with pyranose ring of the cellulose surface. These interactions disrupt the cellulose crystalline structure by swelling cellulose microfibrils, splitting intra- and intermolecular hydrogen bonds and allowing glucan chains to enter into the catalytic tunnel, thereby making cellulose more susceptible to enzymatic attack (Palonen et al. 2004, den Haan et al. 2013).

The increased binding affinity of intact cellulases improves the catalytic action of the enzyme on crystalline substrates, but not amorphous or soluble substrates (Le Costaouec et al. 2013). The presence of a CBM increase the concentration of the enzyme on the surface of the substrate, thus helping the catalytic domain to efficiently target the substrate. Increasing the concentration of cellulases to the surface of insoluble substrate is important for efficient catalytic activity and higher hydrolysis yields. CBM is also suggested to solubilise and free the single glucan chains from the cellulose surface (Palonen et al. 2004). The CBM-cellulose binding affect the two phase cellulase-substrate binding events in bimodular enzymes where adsorption occurs very fast and the cellulases reaches the adsorption-desorption equilibrium

within 10-20 minutes of incubation. However, desorption phase is slow due to strong binding affinities of CBM carrying cellulases (Varnai et al. 2014).

The presence of a CBM has not been shown to increase the catalytic activity of the enzyme, but it has been observed that processive Cel7A with or without a CBM proceed at the same speed along cellulose chains (Palonen et al. 2004, Pakarinen et al. 2014). Cellulases lacking CBM are able to produce almost the same level of hydrolysis yields. The activity and free protein concentration of cellulase lacking CBM increases over the course of hydrolysis due to the depletion of available substrate sites after substrate solubilisation (Pakarinen et al. 2014). Cellulases lacking CBMs also have reduced affinity for cellulose substrates and a slower adsorption rate, but display faster desorption rates compared to their counterparts containing a CBM. A faster desorption rate is particularly beneficial in avoiding halting due to an obstacle and improves enzyme recovery after hydrolysis (Varnai et al. 2014, Pakarinen et al. 2014). Removal of the CBMs in the cellobiohydrolases has an effect on the rate of hydrolysis of crystalline cellulose but no such effect has been reported for amorphous cellulose. Several cellobiohydrolases that lack the CBM such as TeCel7A have lower specific activities compared to their counterparts containing the CBM on crystalline cellulose (den Haan et al. 2013, Ilmen et al. 2011).

Recent research suggests that increased substrate loading results in enhanced binding of the enzyme without CBM to substrate, thereby allowing processive catalysis, which is coupled with faster desorption rate and the possibility of recycling the active enzyme after hydrolysis (Pakarinen et al. 2014). The enzyme available on the surface of the substrate can be enhanced by reducing the amount of water to counterbalance the lack of CBM and this can increase the probability of adsorption of the core enzyme to the substrate and efficient catalysis (Pakarinen et al. 2014). Varnai et al. (2013) showed that the CBM is not required for efficient hydrolysis at increased substrate concentration of up to 10% dry matter (DM). Similar results has also been reported where they compared the hydrolysis efficiency of TrCel7A and *Thermoascus aurantiacus* Cel7A (TaCel7A) lacking the CBM (Larsen et al. 2008); at increased substrate concentration, both enzymes reached about the same level of hydrolysis. The main advantage in using enzymes lacking the CBM is the potential to recover of enzyme that is still active and non-bound after hydrolysis. Enzyme recycling is one of the potential strategies that can be used to reduce enzyme costs during the lignocellulose hydrolysis process. However, enzyme recovery is also hindered by non-

productive binding, inhibition and/or denaturation of the enzyme during hydrolysis (Pakarinen et al. 2014, Palonen et al. 2004) and this will be discussed later in this chapter.

The disadvantage of cellobiohydrolases lacking CBM is that they are prone to inactivation and/or denaturation, which suggest that the CBM also plays a role in enzyme stability. There is evidence that the CBM in TrCel7A thermally stabilizes the enzyme by increasing the overall melting point (Pakarinen et al. 2014). Hall et al. (2011) monitored temperature-induced transitions in full length Cel7A and its separate domains (CD and CBM) and found that the melting point for the CBM (65°C) was higher than that of a full length Cel7A protein (59°C) or the CD (51°C). The CD formed aggregates corresponding with its melting temperature, while unfolding and aggregation for the CBM was induced at temperatures much higher than the melting temperature of the whole protein. This indirectly suggests that CBM plays a role in the thermal stability of the protein.

2.4.4 MECHANISMS OF GLYCOSIDIC BOND HYDROLYSIS

Glycoside hydrolases (GHs) have a common characteristic of cleaving the β-1,4-glycosidic bonds through acid-base catalysis (Naumoff 2011). The cleavage of glycosidic bonds during the hydrolysis process is always stereospecific and is achieved by two catalytic residues of the enzyme, i.e. a proton donor and a nucleophile/base. The hydrolytic cleavage can occur via retention or inversion of the anomeric configuration, depending on the spatial configuration (Fig. 7) (Naumoff 2011, Zhang and Zhang 2013). Generally, one of these two conserved mechanisms is present in all GH families regardless of their choice of substrate. However, exceptions include families GH 4, GH 23, GH 83 and GH 97 that use both the retaining and inverting mechanisms (Vuong and Wilson 2010, Naumoff 2011). Other exceptions reported in literature include the enzymatic hydrolysis of low-molecular weight synthetic substrates and carbohydrate derivatives or analogues that may follow different hydrolysis mechanisms than for natural substrates. GHs exhibits specificity mainly to two elements of substrate structure, namely (i) the configuration of cleaved glycoside bond (α or β) and (ii) the size of the hemiacetyl/ketal of the cleaved monosaccharide residue (Naumoff 2011).

In the inverting mechanism, the catalytic acid donates a proton to the anomeric carbon while a catalytic base from a water molecule removes a proton (Vuong and Wilson 2010). This increases the nucleophilicity of the calalytic base and facilitates its attack on the anomeric

centre. The retaining mechanism is characterised by a two-step displacement process where the general acid/base catalyst first perform as an acid and then as a base in glycosylation and deglycosylation, respectively. In the first step, it donates a proton to the glycosyl oxygen atom while the nucleophile forms an enzyme-sequestered covalent intermediate. In the second step, the deprotonated general acid/base acts as a general base and activates a water molecule that carries out a nucleophilic attack on the glycosyl-enzyme intermediate (Fig. 2.7). These two inversion steps result in the retaining of the stereochemistry at the anomeric centre (Vuong and Wilson 2010). The position of the proton donor is within hydrogen-bonding distance of the glycosidic oxygen in both the inverting and retaining mechanisms. However, the nucleophile/base is in close vicinity of the sugar anomeric carbon (approx. 5.5 Á) in retaining enzymes, while it is more distant in inverting enzymes (approx. 10 Á) (Davies and Henrissat 1995). The longer distance in inverting glycosides is to allow the water molecule into the enzyme active centre during the hydrolysis reaction (Naumoff 2011).

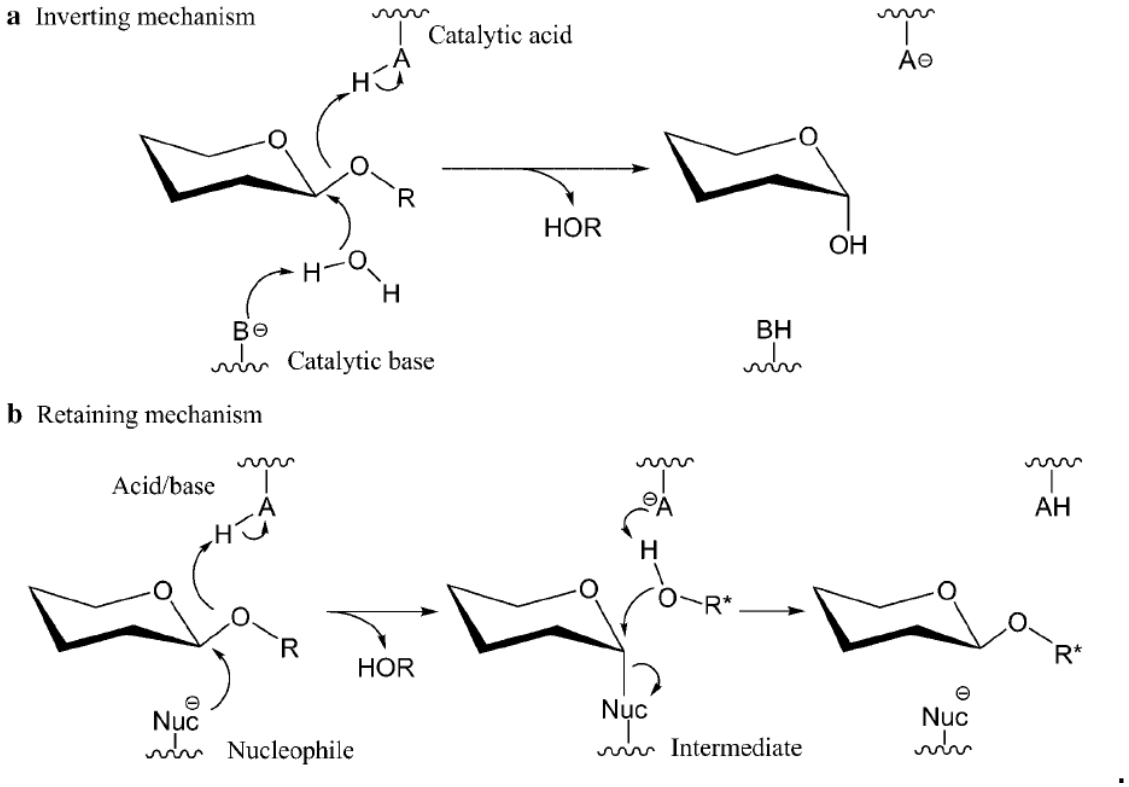

Fig. 2.7: Illustration of the proposed (a) inverting (b) and retaining mechanisms. AH: a catalytic acid residue, B: a catalytic base residue, Nuc: a nucleophile, R: a carbohydrate derivative and HOR (usually a water molecule) an exogenous nucleophile (Vuong and Wilson 2010).

2.5. FERMENTATION: CONVERSION OF LIGNOCELLULOSIC HYDROLYSATE TO BIOETHANOL

To achieve an economically feasible ethanol production process, high cellulosic solid loading of at least 200 g/L is required to yield fermentable sugars with a concentration of at least 100 g/L. The ethanol titres from the fermentation of monomeric sugars must be at least 50 g/L, otherwise lower concentrations result in energy inefficient downstream processing of ethanol. However, increasing the concentration of pretreated solids also results in increased inhibition of enzymatic hydrolysis (Ximenes et al. 2010). Production of ethanol is a multistep process that includes enzyme production, hydrolysis of cellulosic biomass and fermentation of hexose and pentose sugars (Goyal et al. 2011). Successful ethanol production requires saccharification of lignocellulosic material to monomeric sugars before yeast can convert these sugars to ethanol (Fujita et al. 2002, Fujita et al. 2004).

Various ethanol process integration approaches can be pursued to minimise the overall process costs (Lynd et al. 1999, Hasunuma and Kondo 2012) (Fig. 2.8). Separate hydrolysis and fermentation (SHF) involves separate hydrolysis of cellulose material and fermentation of glucose to ethanol in separate reactors. This approach is suitable because both hydrolysis and fermentation have different optimum conditions, but the main drawback to this approach is end-product inhibition of hydrolysing enzymes that rapidly decreases the rate of hydrolysis with increasing concentrations of cellobiose and glucose (Hahn-Hägerdal et al. 2006). This problem can be relieved in simultaneous saccharification and fermentation (SSF) where both hydrolysis and fermentation proceed in one reactor vessel (Hasunuma and Kondo 2012). The continuous conversion of glucose to ethanol prevents the accumulation of glucose, thereby avoiding feedback inhibition. This process can be further upgraded to a simultaneous saccharification and co-fermentation (SSCF), where hydrolysis of both hexose and pentose sugars is achieved in one bioreactor. The major drawback of an SSF configuration is that the optimum temperature for saccharification is at 50ºC, while the optimum temperature for most fermenting microorganisms is between 28ºC and 37ºC (Hahn-Hägerdal et al. 2006, Hasunuma and Kondo 2012).

The ultimate integrated process known as consolidated bioprocessing (CBP) (van Zyl et al. 2007) incorporates multiple biologically mediated ethanol production steps (enzyme production, hydrolysis of cellulose and fermentation of lignocellulose sugars to ethanol) within one microorganism or a microbial consortium in one reactor. Integration of multiple

steps in one bioreactor is foreseen as a potential breakthrough in reducing operational costs by up to four-fold (Lynd et al. 2005, Goyal et al. 2011, Olson et al. 2012). Integration can ensure reduction of energy demands, equipment, cost, time and recirculation of process stream wastewater.

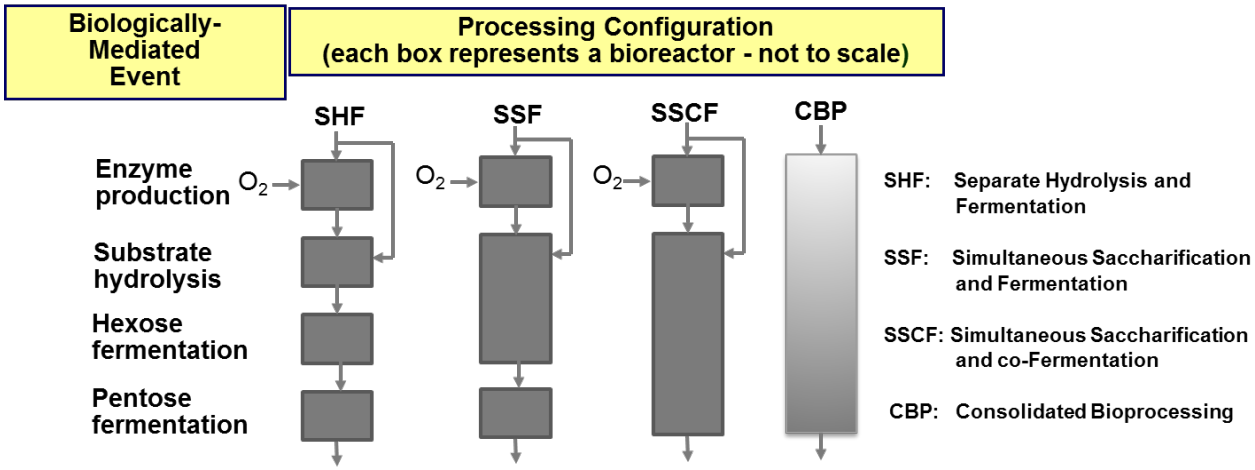

Fig. 2.8: Fermentation configuration modified from Lynd et al. (1999).

The selection of a suitable host that can withstand the metabolic burden of producing multiple enzymes and produce ethanol at titres high enough for the process to be economically efficient is of utmost importance (Jung et al. 2012). The yeast *Saccharomyces cerevisiae* offers many traits such as high ethanol productivity and high ethanol tolerance, which makes it an ideal organism to achieve CBP (Goyal et al. 2011). It is also amenable to genetic manipulation, has a long history with the food and beverage industry, and is generally regarded as safe (GRAS) (Hasunuma and Kondo 2012). One limiting factor is that *S. cerevisiae* is unable to utilise the sugar polymers in cellulosic biomass (Goyal et al. 2011). To bypass the expensive step of producing and adding commercial enzymes, various attempts to engineer cellulolytic ability in an ethanologenic microorganism such as *S. cerevisiae*, have been explored (Fujita et al. 2004, Ilmen et al. 2011, Jung et al. 2012). In addition to the introduction of various complementary enzymes, proper secretion systems have to be engineered in recombinant microbes to achieve high titre production of the extracellular enzyme. In addition, suitable enzyme ratios and enzyme-microbe synergy should also be established to avoid feedback inhibition due to the accumulation of products (Jung et al. 2012).

Fujita et al. (2002) constructed a cellulose-degrading yeast that co-displayed the *Aspergillus aculeatus* Cel3A (AaCel3A) and TrCel5A on the cell surface. The recombinant yeast was able to ferment β-glucan and produce up to 93.3% of the total theoretical ethanol yield with a high productivity of 0.53 g/l/h. However, ethanol productivity on β-glucan was lower than on glucose (1.39 g/l/h), which implied that conversion of β-glucan to glucose by a co-displaying strain was a rate-limiting step. In subsequent co-display attempts, the TrCel5A, TrCel6A and AaCel3A were co-expressed in yeast (Fujita et al. 2004) and the strain achieved 0.45 g/g yield of ethanol, which corresponded to 88.5% of the theoretic yield for 40 hours of fermentation on phosphoric acid swollen cellulose (PASC). Another study by Den Haan et al. (2007b) reported the expression of TrCel7B and the *Saccharomycopsis fibuligera* Cel3A (SfCel3A) in yeast strains, documenting the first report on the hydrolysis of pure cellulose (PASC) by supernatant from a yeast strain co-expressing these cellulases. However, it was apparent that expression of cellobiohydrolases is the most critical step in order to access the crystalline regions of cellulose. Expression of cellobiohydrolases from four ascomycetes in yeast allowed for the hydrolysis of PASC and bacterial microcrystalline cellulose (BMCC), albeit at very low enzyme activity levels (Den Haan et al. 2007a).

The challenge is therefore to achieve sufficient expression quantities and high activity of cellulases for the conversion of both amorphous and crystalline cellulose (Olson et al. 2012). In a recent study, high-level expression and improved activity of two cellobiohydrolases, Cel7A and Cel6A, were reported (Ilmen et al. 2011). The supernatant from *Talaromyces emersonii* Cel7A (TeCel7A) and *Chrysosporium lucknowense* Cel6A (*C.l.*Cel6A) expressed in *S. cerevisiae* displayed the highest activity on Avicel compared to other cellulases. To improve cellulose targeting of Cel7A, a CBM derived from *T. reesei* was fused to the C-terminal domain of the *T. emersonii* Cel7A (TeCel7A-TeCBM). The fusion cellulase showed high Avicel conversion compared to the cellulase without the CBM (Ilmen et al. 2011).

In addition to CBP strain development, commercial scale application of the CBP approach will also require the optimization of bioreactor conditions and proper understanding of their effects on microbial activity. In a bioreactor, the CBP strain would convert lignocellulose hydrolysate in the presence of inhibitory compounds resulting from the pretreatment of lignocellulose biomass (Modig et al. 2008). Therefore, a detailed analysis of the effect of lignocellulosic hydrolysate derived inhibitors on biological elements need to be established.

2.6 INTERACTION OF CELLULASES WITH LIGNOCELLULOSIC HYDROLYSATE-DERIVED INHIBITORS

Various factors can result in the inhibition of cellulolytic enzymes during bioethanol production, including product inhibition, particle size effect, mass transfer limitation, the recalcitrance of the remaining cellulose and xylo-oligosaccharides and more importantly, non-productive adsorption of enzymes by lignin (Alvira et al. 2010, Ximenes et al. 2010, Qing et al. 2010). Lignin confers mechanical strength in the plant cell wall by forming a physical barrier and intercalate in the spaces of the cell wall, thus forming covalent bonds with cellulose and hemicellulose polymers that limits the access of hydrolytic enzymes to sugar polymers (Olsen et al. 2011). The diversity and distribution of phenolic structures within the lignin polymer determine the magnitude of enzymatic hydrolysis inhibition that the lignin will contribute. The removal of lignin from the hydrolysate increases the available surface area, thereby increasing access of cellulases and improving cellulose digestibility (Zheng et al. 2013).

Disruption of lignin results in the release of low-molecular weight phenols that inhibit enzymatic hydrolysis. Both soluble and insoluble lignin interferes with the enzymatic hydrolysis by absorbing cellulases, cellulase precipitation, inhibition and/or deactivation of cellulases (Tejirian and Xu 2011). Residual lignin also interferes with enzymatic hydrolysis and three mechanisms have been proposed: (i) acting as a physical barrier preventing access to cellulose fibers; (ii) inhibition of cellulases by soluble lignin-derived compounds and (iii) adsorption of cellulases onto lignin-rich residues resulting in non-productive binding (Olsen et al. 2011, Rahikainen et al. 2013a)**.**

2.6.1 INHIBITION OF CELLULOSE HYDROLYSIS BY LIGNIN AND LIGNIN-DERIVED PHENOLICS

The effects of lignin and its degradation products on enzymes are highly dependent on enzyme affinity for particular lignin-residues and vary depending on the microbial source of the enzyme (Guo et al. 2014). Research shows that TrCel3A displays stronger affinity for lignin than for carbohydrates (Kaya et al. 2000). As a model polymeric lignin, tannic acid appeared to be a strong soluble inhibitor of cellulases, particularly TrCel3A activity, whereas insignificant effects were observed for the *Asperillus niger* Cel3A (AnCel3A) (Ximenes et al. 2011). The enzyme source-dependence of Cel3A hydrolytic activity was further confirmed using a strong indicator of Cel3A hydrolytic activity, *ρ*-nitrophenyl glucopyranoside (pNPG). Similar effects as those observed with tannic acid were also displayed by gallic acid where

approximately 30% inhibition was observed with pNPG as a substrate for TrCel3A, while no effect was observed on AnCel3A (Ximenes et al. 2011). Haven and Jorgensen (2013) observed that there was no significant decrease in the Cel3A activity from Novozyme 188 on all the substrates tested, whereas Cel3A derived from Cellic CTec2 (an enzyme cocktail based on *T. reesei)* adsorbed more significantly onto lignin and pretreated wheat straw. Recent findings by Ko et al. (2015b) also provided evidence that Cellic CTec derived Cel3A activity was reduced from 17.3 to 2.0% during the hydrolysis of lignin derived from liquid hot water pretreated hardwoods. These findings suggest that hydrophobic interactions between Cellic CTec-derived Cel3A and lignin were most likely the cause of adsorption. These results also highlighted that Cel3A from different sources have different affinities for adsorption to lignin or other hydrophobic surfaces (Haven and Jorgensen 2013).

Haven and Jorgensen (2013) observed a small amount of free activity in solution for the Cel3A derived from commercial Cellic CTec2 preparation, but surprisingly, there was no cellobiose build-up during the hydrolysis process. They hypothesised that the Cel3A enzyme could be adsorbed to the residual solids, but this adsorption did not deactivate Cel3A as it maintained its catalytic activity. Other studies suggested that Cel3A could adsorb to lignin and hemicellulose preparations, but to a smaller extent compared to other cellulases (Pribowo et al. 2012, Varnai et al. 2014). In contrast to these findings, Guo et al. (2014) reported that Cel3A was the least affected by lignin inhibition. They detected that the lack of CBM in Cel3A made this enzyme less likely to adsorb onto lignin residues. This report was supported by earlier studies where the activity of Cel3A did not change during the course of hydrolysis, which suggested that Cel3A remained free in solution (Berlin et al. 2006). One can conclude that the observed adsorption of Cel3A largely depends on the microbial source from which the enzyme is derived.

Endoglucanases are reported to have affinity for both lignin and carbohydrates, while xylanases bind more to carbohydrates than lignin (Kaya et al. 2000). However, other evidence suggested that xylanases and cellulases such as Cel7A, Cel5A have strong affinities for lignin (Guo et al. 2014). In contrast, other studies found Cel5A activity to be decreased only by 9.6% while there was no change in the Cel7A activity (Ko et al. 2015b). Some studies have used commercially available oligomeric phenolics to simulate lignin in lignocellulose hydrolysates, where a concentration of 1 mM tannic acid resulted in ~70-80% hydrolysis inhibition of Avicel hydrolysis (Tejirian and Xu 2011). Comparison of inhibition effect between tannic acid and inhibitor sources containing equivalent amounts of tannic acid

subunits (galloyl and Glc subunits), showed that the inhibition from other compounds were moderate (~20% decrease in the extent of hydrolysis) compared to tannic acid (70% decrease). This suggested that in addition to tannic acid subunits, the structural organisation and complexity of tannic acid plays a major role in initiating the inhibition effect (Tejirian and Xu 2011).

Panagiotou and Olsson (2007) compared the hydrolysis of a synthetic inhibitor cocktail simulated from the wheat straw hydrolysate and the actual wheat straw hydrolysate. The performance of a commercial enzyme cocktail composed of Celluclast-Novozyme or *Penicilium brasilianum* enzyme decreased to between 60% and 80%, whereas the wheat straw hydrolysate resulted in a decrease of up to 85%. The increased inhibition effect of the wheat straw hydrolysate may have been due to additional trace compounds that were present (Panagiotou and Olsson 2007). The synthetic inhibitor cocktail with a total pool of inhibitor compounds was found to decrease the hydrolysis performance of Celluclast-Novozyme mixture and *P. brasilianum* crude extract by 34% and 36%, respectively.

Kim et al. (2011) tested red maple hydrolysate with a solid loading of 220 g/L pretreated substrate using liquid hot water (LHW). The detected inhibitor compounds in maple hydrolysate included 1.3 g/L total phenolics, 0.7 g/L HMF and 3.4 g/L furfural, but formic and levulinic acid were not detected. A 1% solka floc hydrolysis reaction treated with maple hydrolysate was compared to a buffer treated reaction: the maple hydrolysate reduced the hydrolysis rate by 50%; the glucose yield was only 40% of the theoretical maximum and glucose accumulation levelled off in 24 hrs. The hydrolysis of 1% solka floc (with buffer only) with low (1 mg) and high enzyme (25 mg) enzyme loadings resulted in 70% and 92% conversion, respectively. The study revealed that even though low enzyme loadings decrease the hydrolysis rate by 50%, the conversion rate was only decreased by 22%. This provided evidence that a lignin-free cellulase system can be hydrolysed at low enzyme loadings and that inhibition was due to soluble compounds in the maple hydrolysate (Kim et al. 2011).

In lignocellulosic hydrolysates, the inhibition is mainly a combined effect because the inhibitory compounds are present in a mixture (Piotrowski et al. 2014, Panagiotou and Olsson 2007). The complexity of inhibitors in hydrolysates makes it difficult to determine the relative contribution of each potential inhibitor compound towards the inhibition of cellulose-enzyme systems (Kim et al. 2011, Guo et al. 2014). A selective inhibitor removal approach

was reported by Kim et al (2011) who studied the effect of sequentially removing the inhibitors released during LHW pretreatment of red maple. The soluble inhibitors were sequentially removed from the pretreatment liquid by Pluronic L62D, activated carbon or ethyl acetate. Similar concentrations of inhibitors as identified in the maple hydrolysate were introduced either individually or in combination to a 1% solka floc hydrolysis reaction with the aim of mimicking the red maple hydrolysate. The removal of phenolics in maple hydrolysate resulted in the increase of glucose yield of up to 20% and the glucose accumulation continued after 48 h, confirming that phenolic compounds were potent inhibitors in hydrolysate (Kim et al. 2011, Ximenes et al. 2011).

Tejirian and Xu (2011) who added inhibitor compounds present in pretreated corn stover hydrolysate individually at a final concentration of 1 mM to the hydrolysis reaction reported a similar strategy. The monomeric compounds selected were vanillin, coniferyl alcohol, 4-hydroxyl-2-methylbenzoic acid, coniferyl aldehyde, ferulic acid, syringyladehyde, ellagic acid and flavonol. Individually added monomeric phenols did not show significant inhibition effects. When the concentration of vanillin and 4-hydroxyl-2-methylbenzoic acid was increased to 10 mM (i.e. beyond the values reported in literature), hydrolysis performance was drastically reduced by approximately 70%. Further increases in the concentration of vanillin to 500 mM resulted in the gelation of the reaction suspension and subsequently caused the hydrolysis reaction to terminate (Tejirian and Xu 2011). Concentrations of 1 mg/mL vanillin or 4-hydroxybenzaldehyde previously resulted in a decrease of 7% and 2% in glucose production respectively (Jing et al. 2009). Ximenes et al. (2010) also reported that only vanillin displayed strong inhibition of all cellulases, whereas syringaldehyde and trans-cinnamic acid displayed moderate inhibition. The least inhibition effect was displayed by 4-hydroxybenzoic acid.

2.6.2 INHIBITION OF CELLULOSE HYDROLYSIS BY FURAN ALDEHYDES AND ALIPHATIC ACIDS

The hemicellulose sugar components typically contain acetyl groups amounting 1-6% of total dry weight (w/w), depending on the plant species (Jönsson et al. 2013). These acetyl groups are suggested to play a role in the mechanism of cell wall resistance to enzymatic hydrolysis by interfering with enzyme recognition and thus slowing the rate of hydrolysis. Deacetylation of the hemicellulose backbone improves the enzymatic digestibility of biomass (Zheng et al. 2013, Jönsson et al. 2013, Almeida et al. 2007). Investigation of the effects of acetic acid on the production of cellulases by *T. reesei* RUT C30 found that increasing the concentration of acetic acid did not affect the production of cellulases; instead, Cel3A production increased

with increasing acetic acid concentration. The medium used to grow the recombinant strain was adjusted to pH 6, which may have minimised the pH-depended inhibition effects of acetic acid (Szengyel and Zacchi 2000). Furthermore, LHW-pretreated hydrolysates are known to contain lower concentrations of acetic acids, which do not show a strong inhibition effect during enzymatic hydrolysis (Kim et al. 2011, Ko et al. 2015a).

In a study by Panagiotou and Olsson (2007), formic acid was identified as a strong inhibitor of enzyme hydrolytic performances. Treatment with 4 g/l formic acid resulted in an 80% decrease in glucose concentration produced; increasing the concentration to 15 g/L almost totally inactivated the enzymes. The concentration of formic acid in wheat straw with solid loadings of 60 g/L was approximately 4.7 g/L and increasing the dry matter of wheat straw hydrolysate to a high loading of 220 g/L also increased the concentration of formic acid (Erdei et al. 2010, Herrera et al. 2004). However, Jing et al. (2009) reported that treatment of cellulose hydrolysis with 25 mg/ml formic acid only resulted in a 20% decrease in glucose accumulation.

Enzymatic hydrolysis at 50°C for 60 min in the presence of 3 mg/mL of either furfural or 5-HMF in 50 mM citrate buffer (pH 4.8) reduced glucose concentration by 5% and 10%, respectively, indicating that HMF was a stronger inhibitor than furfural (Jing et al. 2009). The inhibition effect of furfural was also reported by Szengyel and Zacchi (2000) where the addition of 1.2 g/L furfural to a cellulosic fermentation resulted in an approximately 50% decrease in the production of cellulases by the yeast strain. LHW-pretreated hydrolysate contained low levels of furfural, which did not induce an inhibition effect in enzymatic hydrolysis (Kim et al. 2011).

Taken together, the above-mentioned results show that the effect on enzymatic hydrolysis due to the presence of lignin, lignin residues and degradation products resulting from sugar degradation varies depending on the type of enzyme, source of enzyme and the synergistic relationship between the inhibitory compounds in hydrolysate. These factors have a negative impact on the hydrolysis rate. Since more studies were aimed at the impact of lignin and lignin residues, there is more evidence that suggest that lignin and its residues have a strong inhibition effect towards cellulases. However, some evidence suggests that other compounds such as weak acids and furans are also strong inhibitors of enzymatic hydrolysis. In addition, the effect of some compounds may be insignificant when added individually, but when in synergy with other compounds they might initiate a strong inhibition

effect. The synergistic relationship between hydrolysate compounds is very complex since the content of these compounds in hydrolysate varies depending on a number of factors such as biomass type, pretreatment severity, etc. Therefore, it is nearly impossible to define this phenomenon. One possible strategy is to clearly define the role of individual compounds on a wide spectrum of enzymes and design pretreatment strategies that result in the elimination or reduction of those compounds identified.

2.6.3 NON-PRODUCTIVE ADSORPTION OF CELLULASES ONTO LIGNIN

The binding of cellulases onto lignin is not only dependent on the lignin content, but is also influenced by the physical and chemical nature of lignin residues (Gao et al. 2014). These physical and chemical properties of lignin moieties mediate the non-productive adsorption of cellulases onto lignin, thereby reducing the efficiency of these enzymes in hydrolysing lignocellulosic biomass. The lignin-carbohydrate network in lignocellulosic biomass creates steric hindrance and decreases the movement of cellulases within glycan chains (Fig. 2.9) (Palonen et al. 2004, Varnai et al. 2011). The biomass source, pretreatment strategy and conditions, structural and chemical properties of lignin resulting from the pretreatment strategy and conditions used affect the capacity and extent of enzyme adsorption onto lignin (Zheng et al. 2013, Guo et al. 2014). Lignin from dilute acid catalysed steam explosion pre-treatment adsorbs cellulase enzymes more strongly than steam explosion or LHW pretreatments (Ko et al. 2015a).

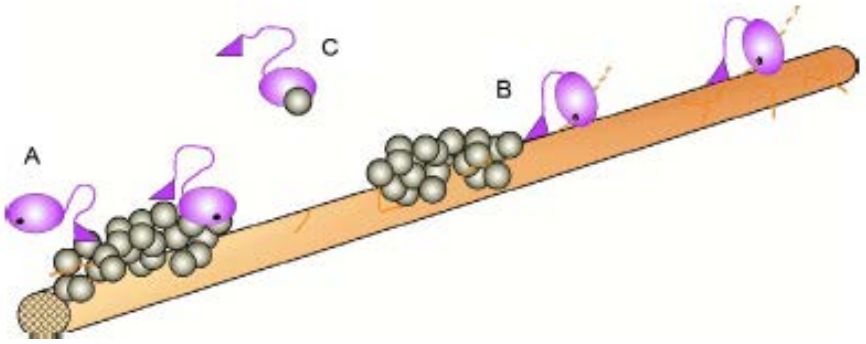

Fig. 2.9: Inhibitory mechanisms of lignin in enzymatic depolymerisation of cell wall carbohydrates (A) enzyme adsorption onto lignin (B) restriction of enzyme accessibility into the carbohydrates and (C) enzyme inhibition by soluble lignin-derived compounds (Rahikainen et al. 2013a).

Non-productive binding is an extensively studied phenomenon, but the exact mechanism remains unresolved (Nakagame et al. 2011, Rahikainen et al. 2013a). Non-productive

binding of cellulases onto lignin and lignin residues can result in inhibition and/or deactivation of the enzyme. There are three types of interaction that contribute to non-productive adsorption, namely (i) hydrophobic forces (Eriksson et al. 2002); (ii) hydrogen bonding (Berlin et al. 2006, Zheng et al. 2013); and (iii) electrostatic interactions (Berlin et al. 2006). Hydrophobic interactions are the dominant forces driving non-productive adsorption of cellulase enzymes to lignin (Varnai et al. 2014).

(i) Hydrophobic interactions

The hydrophobicity of lignocellulose degradation compounds is mostly correlated with their inhibitory potential, i.e. the extent to which the hydrophobic residues of the enzymes or proteins bind through hydrophobic interactions (Klinke et al. 2004). Moieties such as carboxyl groups (more hydrophilic) affect enzyme adsorption less than phenolic hydroxyl groups (more hydrophobic) (Guo et al. 2014). Nakagame et al. (2011) showed that lodgepole pine lignin subjected to steam pretreatment had less affinity for cellulases than the organosolv pretreated lignin. They further used solution-state NMR that showed that the steam-pretreated lignin had an increased content of aliphatic carboxylic acid groups that could have increased the hydrophilicity and negative charge of this lignin and thus decrease its binding affinity for cellulases. Further evidence that different moieties on the surface of the protein contribute to non-productive binding has been shown using atomic force microscopy (AFM). Qin et al. (2014) used specialised atomic force microscopy (AFM) tips coated with either carboxylic or hydroxyl groups and reacted them with immobilised enzymes. They showed that the hydrophobic forces were stronger with the carboxylic groups coated tips than with the tips coated with the hydroxyl groups. These results gave an overview of the real situation even though the enzymes were immobilised whereas in real conditions they are in a free system. The increased carboxylic acid groups in the lignin surface may also facilitate solubilisation of lignin in water and thus enhance the enzymatic hydrolysis of pretreated substrate (Nakagame et al. 2011).

Substitution of methoxy groups located *ortho* to the phenolic hydroxyl group has been shown to reduce the toxicity of phenols towards *S. cerevisiae* (Klinke et al. 2004). The hydrophobicity of phenols can be drastically reduced by the introduction of a methoxyl group in the aromatic ring and this reduction is less dependent on the functional group *para* to the phenol hydroxyl group (Klinke et al. 2004). Other chemical modifications can include increasing the amount of coniferyl alcohol, synapyl alcohol, ferulic acid and carboxylic acid content in lignin. This should increase lignin hydrophilicity and consequently improve the

enzymatic hydrolysis of lignocellulose substrates (Nakagame et al. 2011). In addition to chemical modifications, genetic modification of plant biomass source can yield crops with increased content of desired chemical groups (Bonawitz et al. 2014, Ko et al. 2015a). Hydrophobicity can be used as a tool for comparison between different functional groups. The degree of inhibition is generally higher in phenolic aldehydes and ketones compared to phenolic acids. However, in phenols with the same amount of methoxyl groups, it is difficult to use hydrophobicity as a tool for differentiation given very small differences in hydrophobicity (Klinke et al. 2004).

Recent studies found that both the surface properties of the catalytic core in cellulase enzymes and the hydrophobic amino acid residues in CBM might also be responsible for a high affinity to cellulose and therefore play a role in non-productive binding onto lignin (Varnai et al. 2014). Gao et al. (2014) suggested that reducing the hydrophobicity of lignin could help in recovering cellulase enzymes such as Cel6A. Predictions of hydrophobic patches using computational modelling suggested that the hydrophobicity amongst cellulases decreases from Cel6A>Cel7A>Cel7B. However, experimental evidence did not support these computational findings, which further suggest that other factors - in addition to hydrophobic interactions - facilitate lignin-enzyme binding. In particular, it was noted that Cel7B was the least recovered compared to exo-cellulases and it was ascribed to an open-cleft in the active site of the Cel7B that allows easy access for lignin residues to aromatic amino acids and thereby facilitating hydrophobic interactions with lignin. In addition to determining the activities that are impacted by lignin residues, it is also critically important to determine which enzymes are prone to inhibition and/or deactivation by non-productive binding to each type of lignin residue in pretreated hydrolysate (Gao et al. 2014).

(ii) Hydrogen bonding

The phenolic and aliphatic hydroxyl groups significantly contribute to non-productive adsorption by forming hydrogen bonds between cellulases and lignin (Rahikainen et al. 2013a). The carboxylic acid groups in lignin have been shown to decrease the hydrophobicity of lignin structures and thus reducing the negative effects of lignin (Rahikainen et al. 2013a, Zheng et al. 2013). The acetyl groups are suggested to inhibit the formation of hydrogen bonds between cellulose and the catalytic domains that are required for productive binding, and may increase the diameter of the cellulose chain, which increases steric hindrance (Zheng et al. 2013).

(iii) Electrostatic interactions

Physical parameters such as electrostatic interactions also play a role in enhancing non-productive adsorption (Rahikainen et al. 2011, Varnai et al. 2014). In electrostatic interactions, the charged or partially charged proteins adsorb to lignin surfaces, which is suggested to change the protein conformational structure and intensify the protein-surface interaction. The binding is facilitated by hydrophobic and electrostatic interactions and may increase, depending on the pH levels (Rahikainen et al. 2011, Rahikainen et al. 2013b). Other studies also showed that Cel3A adsorption to lignin is highly depended on the pH (Ko et al. 2015b). Lignin carries negatively charged groups that increases in number with increasing pH levels. The positively charged group on the enzyme surfaces binds non-specifically onto negatively charged groups in lignin, suggesting that electrostatic interactions also play a role in non-productive binding (Varnai et al. 2014). It is believed that the negative charge imparted by an increased number of carboxylic acid groups on the surface of steam-pretreated lignin may decrease enzyme binding by electrostatic repulsion due to negative charges on the cellulase enzyme surface (Nakagame et al. 2011, Strobel et al. 2015). The contribution of the CBM domain to non-productive adsorption is also dependent on the pH and the type of lignin compound. However, the role of smaller CBM domains in lignin binding may be insignificant at lower pH if the CD is large and capable of binding to lignin (Rahikainen et al. 2013a).

A temperature increase from 25°C to 45°C enhanced the desorption rate of cellulases while further increases rapidly dropped the desorption rate, indicating that temperature plays a role in the hydrophobic interactions (Nakagame et al. 2011). The changes in temperature and pH may promote enzyme desorption, but may also cause deactivation and denaturation of enzymes at a certain level (Nakagame et al. 2011, Varnai et al. 2013). The enzyme-lignin interactions are more pronounced at temperatures from 45°C and higher, suggesting that protein unfolding is also facilitated by an increase in temperature that causes the protein to lose its structure and become irreversibly bound to lignin surfaces and the subsequent loss of activity (Rahikainen et al 2011, 2013b). These authors found that TrCel7A was inhibited by lignin at a temperature of 45°C, whereas TeCel7A derivatives (one fused with a family 3 CBM and the other with a family 1 CBM) were inhibited by lignin at 55 °C and 65 °C, respectively. Both the TeCel7A cellullases possess the same thermo-stabilities and the differences in their temperature-induced lignin inhibition could only be attributed to the different CBMs fused to the enzymes. The inhibitory effect on TrCel7A and TeCel7A-CBM1 was highly different although both these enzymes shared a CBM, a linker peptide and 66% sequence similarities, suggesting that fusion of the family 1 CBM conferred thermo-stability

to the *T. emersonii* enzyme (Rahikainen et al. 2013b). These findings suggested that a thermostable enzyme with a rigid structure could efficiently perform hydrolysis due to its inherent resistance to unfolding. The possible means of avoiding protein denaturation at elevated temperatures during hydrolysis is to reduce the process temperature or to use surface-coating agents such as non-ionic surfactants (Rahikainen et al, 2011).

To better understand the effect of non-productive enzyme binding and to prevent or minimise adsorption effects, the interactions between cellulases and lignin should be evaluated under varying conditions (pH, temperature and ionic strength) relevant to the real fermentation scenario (Qin et al. 2014). The impact of non-specific adsorption can be alleviated by three proposed strategies: (i) coating lignin surfaces with lignin-blocking agents; (ii) choosing pretreatment technologies that result in hydrolysates with minimum binding affinities; and (iii) screening or engineering enzymes with lower lignin binding affinities. Research shows that the level of binding of cellulases onto lignin is to some extent dependent on the source of the enzyme. However, more research is required to understand the interactions involved in the non-specific enzyme adsorption to lignin (Varnai et al. 2014). There is little or no information on the possible binding of other lignocellulose hydrolysate-associated inhibitors such as furfural, HMF or organic acids. Information on these pretreatment by-products is limited to its inhibition and/or deactivation effect on cellulases (Ximenes et al. 2010, 2011, Tejirian and Xu, 2011, Kim et al. 2011, 2015).

2.7. LIGNIN BLOCKING/MODIFYING AGENTS

Various agents and strategies have been proposed to minimise non-productive adsorption or even modify the surface of lignin residues to alleviate cellulase adsorption onto lignin or lignin residues (Alriksson et al. 2011, Borjesson et al. 2007). Addition of surfactants, polymers and non-catalytic proteins such as bovine serum albumin (BSA) was shown to improve enzymatic hydrolysis of lignocellulose substrates. Surfactants are suggested to adsorb to lignin surfaces and therefore prevent unspecific binding of enzymes onto lignin residues. The suggested mechanism is that they bind to lignin through hydrophobic interactions while the hydrophilic region creates steric hindrances that block the enzymes from non-specific binding to lignin (Borjesson et al. 2007, Sammond et al. 2014). The hinderance role increased with increasing length of the hydrophilic chain in polymers such as ethylene oxide and was more pronounced in highly crystalline cellulose substrates (Borjesson et al. 2007).

Non-ionic surfactants have been identified to be the most effective in preventing non-specific adsorption of enzymes (Eriksson et al. 2002). The effect of poly-ethylene glycol (PEG) was shown to be specific for certain monocomponent cellulases: a 45% improvement in the hydrolysis was observed with TrCel7A while only a 1% improvement was observed with TrCel5A in the presence of PEG. Surfactants such as PEG were suggested to stabilise the cellulases, particularly preventing enzyme unfolding and thermal denaturation of TrCel7A and thus allowing for enzymatic hydrolysis at higher temperatures (Hsieh et al. 2015). However, the addition of surfactants or BSA to cellulose in the absence of lignin did not substantially change the conversion of cellulose, implying that they do not impact the catalytic activity of the enzyme (Eriksson et al. 2002, Borjesson et al. 2007).

Kim et al. (2015) reported that the adsorption of cellulases onto lignin is more pronounced at lower enzyme loading, but the adsorption effect at high enzyme loadings might be mitigated by an excess amount of (free) enzyme available for cellulose hydrolysis lignin. The addition of additives such as BSA can potentially reduce the enzyme loading required for efficient hydrolysis (Kim et al. 2011, Sammond et al. 2014), but BSA is expensive and high levels are required to displace cellulase binding onto lignin. More research is required to investigate other less expensive additives that can be potentially used as blocking agents to reduce the enzyme loadings for efficient biomass hydrolysis (Kim et al. 2015).

Various detoxification strategies as reviewed in (Mussatto and Roberto 2004, Palmqvist and Hahn-Hagerdal 2000a) have been proposed to decrease enzyme inhibition during cellulosic conversions. However, these strategies have focused on the fermenting microorganism with little attention paid to the enzyme performing the hydrolysis (Jönsson et al. 2013). Since lignocellulose conversions involve both enzymatic hydrolysis and fermentation, there are good reasons to address the inhibition problem of both these processes in combination rather than as separate entities. This is particularly important in configurations such as SSF or CBP where both enzymatic hydrolysis and fermentation proceed in one reactor. Chemical detoxification using reducing agents *in situ* has been recently described as a powerful strategy to address the inhibition problem for both the enzymes and microbial catalysts (Alriksson et al. 2011, Soudham et al. 2011, Cavka and Jonsson 2013). These reducing agents - such as sodium dithionite (sulphite) - interact with lignin residues and sulfonate the inhibitors, changing their chemical form and thus rendering them more hydrophilic and unreactive (Jönsson et al. 2013, Cavka and Jonsson 2013). The advantages of using reducing agents include that there is no need for pH adjustment, they do not react with

sugars and they are compatible with SSF or CBP, which eliminates the need for a separate detoxification step (Jönsson et al. 2013, Alriksson et al. 2011).

2.8 CURRENT TECHNIQUES USED IN ENZYME-LIGNIN BINDING STUDIES

Generally, proteins have a hydrophobic core and a hydrophilic surface that interacts with the substrate surface. However, most cellulase enzymes are relatively rich in hydrophobic amino acids that are randomly or uniformly distributed on the protein surface. In addition, the surface of cellulases are less polar than water, rich in functional groups such as hydroxyl and carboxyl groups that facilitate the formation of multiple bonds including hydrogen bonds, charged or partially charged interactions (Nakagame et al. 2010, Nakagame et al. 2011). All these surface characteristics of cellulases are suggested to contribute to both productive binding to cellulose surfaces and non-productive binding to other surfaces such as lignin or lignin residues. Studies on non-productive binding of cellulase enzymes to lignin have focused on overall adsorption throughout the enzymatic hydrolysis reaction (Rahikainen et al. 2011, Qin et al. 2014).

The rapidly growing field of proteomics and structural genomics is supported by the development in analytical techniques such as circular dichroism (CD) spectroscopy, differential scanning calorimetry (DSC), differential static light scattering (DSLS) and differential scanning fluorimetry (DSF) (Greenfield 2006, Vivoli et al. 2014). These techniques can measure melting temperature changes in the protein bound to a ligand. The melting temperature (T_m) is defined as the temperature at which the native and the denatured states are equally present at equilibrium. This property of the protein is highly dependent on its folding and unfolding status (Seo et al. 2014).

Differential scanning fluorimeter (DSF) is gaining wide recognition as a tool in structural biology and biophysics (Seabrook and Newman 2013). This technique, also known as the thermal shift assay or thermofluor, was first described in 2001 for application in drug discovery and is used as a screening tool for developing working formulations by measuring thermal stability under different conditions (Seabrook and Newman 2013, Vivoli et al. 2014). It is a low-cost technique and utilizes widely available RT-PCR hardware for thermal analysis. The dyes used for DSF bind to hydrophobic core of an unfolding protein in an aqueous environment where the fluorescent agent is quenched. Sypro® orange is a

commonly used dye in DSF due to its specific property of only activating fluorescence when binding to exposed hydrophobic patches (Vollrath et al. 2014).

During DSF, the protein starts in a properly folded state where there is little interaction between dye and folded protein. The protein unfolds as the protein-dye mix is heated, exposing hydrophobic regions where dye binds and the protein starts to fluoresce. The fluorescence increases as the protein continues to unfold until its chains aggregate, excluding the dye in the process (Fig. 2.10). The DSF allows for the identification of individual traits that can be used to probe the stability of a protein under different conditions, e.g. a higher T_m is associated with increased stability (Vivoli et al. 2014, Rosa et al. 2015). In most proteins, stability decreases with increasing temperature, which also decreases the Gibbs free energy of unfolding, ΔG_u until it becomes zero at equilibrium. The equilibrium is reached when the concentration of unfolded and folded proteins is equal and this is termed the melting temperature (T_m). The equilibrium thermodynamics apply if the protein unfolds in a reversible two-state manner. Binding of a ligand to a protein typically results in the increasing of ΔG_u and this may increase the T_m. In most proteins, unfolding is not a reversible two-state reaction (Niesen et al. 2007).

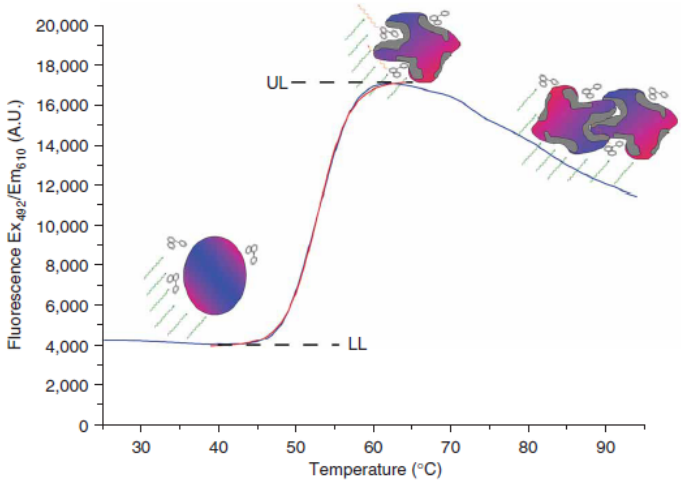

Fig. 2.10: Representation of a three state transition-melting curve (DSF) of a protein (Niesen et al. 2007). UL indicate the upper level and LL indicate the lower level.

2.9 CONCLUSIONS AND REMARKS

The inhibition strength and concentration of lignocellulose degradation products is inversely correlated with the compounds present at a very low concentration, such as furfural and phenolic compounds being stronger inhibitors than acetic acid and levullinic acid (Jing et al. 2009). Understanding the properties of lignin that enhance non-productive adsorption of cellulases can help in the design of pretreatment processes, engineering enzymes with reduced lignin-binding affinity. It can also assist in selective removal of specific compounds in hydrolysates and/or genetic modification of plant biomass with a reduced occurrence of these properties and allow for the high production levels of the desired products (Panagiotou and Olsson 2007, Guo et al. 2014). These strategies are invaluable for the development of cost-effective biofuel production processes. Different analytical methods and findings in literature on the effect of phenolic compounds on cellulases also make it difficult to draw conclusions (Kim et al 2011). Furthermore, high diversity of phenolic compounds hinders the establishment of qualitative and quantitative methods for analyses and therefore mechanisms of inhibition by these compounds are not clearly elucidated (Almeida et al. 2007, Jönsson et al. 2013).

The lack of appropriate techniques to track the binding of individual cellulases within a complex protein mixture onto lignin during enzymatic hydrolysis makes it difficult to understand the extent of non-productive adsorption for individual enzymes. Secondly, it is difficult to distinguish between non-productive binding of cellulases to lignin and productive binding to specific glycans. Research conducted using pure cellulose (eg. Avicel) and purified lignin components can be used to mimic lignocellulose hydrolysis, however, the information acquired from these studies is limited since lignin ultrastructure is modified during isolation and pretreated biomass ultrastructure cannot be simulated. Furthermore, previous approaches have used crude proteins to track binding of cellulases to insoluble biomass, which make it difficult to understand the affinity of individual enzymes to lignin (Gao et al. 2014). In order to find a breakthrough in lignocellulosic hydrolysate conversion, it is important to address the inhibition factors that impact enzymatic hydrolysis (Jönsson et al. 2013).

The purpose of this study was to investigate the impact of individual inhibitory compounds typically released as by-products during pretreatment processes on recombinant cellulases. Chapter 2 is comprised of a comprehensive assessment of the literature background that covers the scope of the work investigated this study. Firstly, we introduce the review with an

overview of biofuels production processes and advances made thus far. This is followed by a detailed discussion of the structural and chemical composition of lignocellulosic biomass. The next section covers the pretreatment strategies and a detailed discussion on inhibitory compounds that are released as pretreatment by-products. Chapter two also includes a detailed review of recombinant cellulase production, cost, structural properties of cellulases and mechanisms of hydrolysis. This section is followed by a comprehensive review on the interaction of cellulases with pretreatment by-products as well as the proposed mechanisms involved in these interactions. The last section is an overview of the relevant techniques used to study protein binding.

The gaps identified in the literature led to questions addressed in the subsequent chapters. Chapter 3 consists of a systematic study, conducted to identify individual inhibitors that strongly inhibit cellulases. This was prompted by the lack of detailed information on the inhibition/deactivation mechanisms for individual cellulases. The inhibitors were selected to represent the three main groups of inhibitor compounds released during pretreatment i.e. furanaldehydes (furfural and HMF), weak acids (acetic acid and formic acid) and phenolic compounds (syringaldehyde, coniferyl aldehyde, cinnamic acid, hydroxybenzaldehyde, hydroxybenzoic acid, acetophenone, tannic acid). We identified a selective inhibition/deactivation effect, which depended on the type of cellulase and the interacting inhibitor compound chemistry. We then selected the identified inhibitor compounds with a strong effect and proposed a detoxification strategy in Chapter 4. Detoxification strategies using sulfur oxyanions or laccase in Chapter 4 were investigated with individual inhibitor compounds in pure cellulose or pretreated sugarcane bagasse fraction.

In Chapter 5, we attempted to identify the mechanism involved in the observed inhibition/detoxification effects. A newly described technique, differential scanning fluorimeter (DSF) which is a promising high throughput tool for studying protein binding and stabilities was selected. Inhibitor compounds that were identified in Chapter 3 to be strong inhibitors/deactivators of cellulases were selected for this study. Some of the inhibitors such as tannic acid were excluded because they interfered with the fluorescence dye used in the technique.

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Chapter 3

RESEARCH RESULTS I

Lignocellulosic Hydrolysate Inhibitors Selectively Inhibit/Deactivate Cellulase Performance

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Lignocellulosic hydrolysate inhibitors selectively inhibit/deactivate cellulase performance

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ABSTRACT

In this study, we monitored the inhibition and deactivation effects of various compounds associated with lignocellulosic hydrolysates on individual and combinations of cellulases. Tannic acid representing polymeric lignin residues strongly inhibited cellobiohydrolase 1 (TeCel7A-TrCBM) and β-glucosidase 1 (SfCel3A), but had a moderate inhibitory effect on endoglucanase 2 (TrCel5A). Individual monomeric lignin residues had little or no inhibitory effect on hydrolytic enzymes. However, coniferyl aldehyde and syringaldehyde substantially decreased the activity of TeCel7A-TrCBM and deactivated SfCel3A. Acetic and formic acids also showed strong inhibition of SfCel3A but not TeCel7A-TrCBM and TrCel5A, whereas tannic, acetic and formic acid strongly inhibited a combination of TeCel7A-TrCBM and TrCel5A during Avicel hydrolysis. Diminishing enzymatic hydrolysis is largely a function of inhibitor concentration and the enzyme-inhibitor relationship, rather than contact time during the hydrolysis process (i.e. deactivation). This suggests that decreased rates of hydrolysis during the enzymatic depolymerisation of lignocellulosic hydrolysates may be imparted by other factors related to substrate crystallinity and accessibility.

Keywords: Lignocellulosic hydrolysate, Inhibition, Deactivation, Cellulases

3.1 INTRODUCTION

The conversion of lignocellulose biomass to ethanol is typically preceded by one or more pretreatment steps to improve access to the fermentable sugars entrapped in the lignocellulose biomass. Enzymatic hydrolysis of the sugar polymers and fermentation of the released sugars yield bioethanol that can be used in the production of transport fuels (Lynd et al. 2002, Martin and Jönsson 2003, Goyal et al. 2011). After capital and feedstock costs, enzymes are the third largest cost component associated with cellulosic ethanol production (Stephen et al. 2012). Lignocellulose is a recalcitrant, but abundantly available biomass composed of three major polymers, namely cellulose, hemicellulose and lignin (Jönsson et al. 2013). Various technologies are available to fractionate sugar polymers (cellulose and hemicellulose) and remove these from lignin, which binds the polymer network together to form a physical barrier that protects the plant cell wall from enzymatic degradation and pathogenic attack (Guo et al. 2014, Mussatto and Roberto 2004). However, in addition to hydrolysable sugar polymers, these pretreatment steps also release various by-products that inhibit cellulases and prevent free access to the cellulose polymer (Jönsson et al. 2013, Almedia et al. 2007).

Glycoside hydrolases (GHs) are classified into different families based on their amino acid sequences and crystal structures. GHs such as cellulases hydrolyse β-1,4-linked cellulose chains in cellulosic biomass using either a retaining or inverting acid-base mechanism (Zhang and Zhang 2013). Complete hydrolysis of cellulose requires the synergistic cooperation of cellulases, which include (1) endoglucanases (1,4-β-D-glucan 4 glucanohydrolases; EC 3.2.1.4), (2) glucanohydrolases including cellobiohydrolases (1,4-β-D-glucan cellobiohydrolases; EC 3.2.1.91), and β-glucosidases (β-glucosidase glucohydrolases; EC 3.2.1.21), to hydrolyse cellulose fibrils to simple sugars (Gao et al. 2014, Kumar and Murthy 2013, Fujita et al. 2002, den Haan et al. 2007). Endoglucanases are non-processive and have groove-shaped catalytic sites that allow the enzyme to randomly cleave amorphous cellulose regions and generate reducing and non-reducing ends. The reducing and non-reducing ends serve as binding sites for cellobiohydrolases (CBHs) where celobiohydrolase 1 (EC 3.2.1.176) and cellobiohydrolase 2 (EC 3.2.1.91), respectively bind and cleaves the crystalline cellulose regions producing cellobiose as a major product (Fujita et al. 2002). The cellobiohydrolases have a tunnel-shaped catalytic site formed by disulphide bridges where β-glycosidic bonds are threaded sequentially and processively (den Haan et al. 2013, Varnai et al. 2014). Cellobiose is subsequently hydrolysed by β-glucosidase which has a pocket-shaped active site that binds non-reducing

glucose units in cellobiose and cleaves them off to produce glucose (Zhang and Zhang 2013).

The binding affinity of lignin residues and other inhibitor compounds to cellulases is enhanced by certain inhibitor and enzyme properties (Guo et al. 2014). The structural and chemical nature of lignin plays a central role in the toxicity of lignin or associated inhibitor residues by increasing the affinity of lignin for cellulases. Rahikainen et al. (2011) reported that lignin prepared by acidic treatment had a higher affinity for endoglucanase, exoglucanase and β-glucosidase than enzymatically prepared lignin. There are also reports that pretreatment changed the lignin surface properties such that it selectively adsorbed some enzyme components more strongly than others (Ko et al. 2015). The high affinity of cellulases for ligneous surfaces prevents the free movement of enzymes that is necessary for the efficient degradation of insoluble lignocellulose sugar polymers. Lignin residues (such as tannic acid, vanillin and syringaldehyde) act as a steric hindrance to cellulolytic enzymes, thus shielding the enzyme away from the cellulose substrate. Non-productive adsorption of enzymes to lignin also makes enzyme recycling difficult as most enzymes will remain bound to lignin, resulting in low enzyme recoveries (Palonen et al. 2004, Kim et al. 2013). Non-productive adsorption of enzymes onto lignin can be prevented by using additives such as various surfactants (Eriksson et al. 2002) and bovine serum albumin (BSA) (Kim et al. 2015) which also enhanced enzymatic hydrolysis. In addition, the additives were suggested to interact with cellulases, increasing their thermal stability and changing the adsorption parameters of cellulases which facilitated enzyme desorption, reducing enzyme loss through irreversible binding (Hsien et al. 2015).

Various compounds contained in the pretreated hemicellulose fraction of lignocellulosic hydrolysates play a major role in the inhibition/deactivation of the hydrolytic enzymes (Tejirian and Xu 2011, Ximenes et al. 2011, Ximenes et al. 2010). Kim et al (2011) defined inhibition as the reversible, instant and rapid binding equilibrium between inhibitors and enzymes and deactivation as the irreversible exponential decrease of enzyme activity with an increase in contact time between inhibitor and enzyme. Little is known about the impact of weak acids and furans on free cellulases since studies have focused only on the effect of these compounds in the microbial production of cellulases rather than the activity of free cellulases. Szengyel and Zacchi (2000) showed that acetic acid had no inhibition effect on *Trichoderma reesei* cellulase activities, but increasing acetic acid concentrations improved SfCel3A activity. Similar results on cellulases were reported by Qi et al. (2014), except that

acetic acid had an inhibitory effect on β-glucosidase. Formic acid is also known as a potent inhibitor of hydrolysis and has been shown to decrease glucose production, while increasing concentration of furfural inhibited both cellulase and β-glucosidase activities (Arora et al. 2013).

Reducing the cost of enzymatic hydrolysis is critical for the economic viability of ethanol production and can be achieved by reducing or eliminating inhibitor compounds in the hydrolysate that lead to non-productive binding, inhibition and inefficient enzymatic hydrolysis. However, it remains difficult to identify the most toxic hydrolysate compounds as previous research reported contrasting conclusions on which compounds play a major role in inhibition or deactivation. This is mainly due to the lack of standard and appropriate techniques to track the impact of lignin and associated hydrolysate inhibitors on individual cellulases within a crude enzyme mixture (Gao et al. 2014). Most inhibition/deactivation studies used inhibitor concentrations higher than those actually found in the different hydrolysates and also used crude commercial enzymes or enzyme mixtures that make it difficult to draw conclusions on the effects of different inhibitor compounds on individual enzymes (Tejirian and Xu 2011, Ximenes et al. 2011, Ximenes et al. 2010).

In this study, we used a systematic approach to investigate the role of individual lignocellulose-associated inhibitor compounds in the inhibition and/or deactivation of TeCel7A-TrCBM, TrCel5A and SfCel3A, either individually or in combination. This study reports on the inhibition/deactivation effects of a broad spectrum of individual inhibitors broadly classified as weak acids, furans and lignin residues on partially purified cellulase components. This study elucidates for the first time, the impacts of weak acids and furans on purified cellulase components.

3.2 MATERIALS AND METHODS

To quantify the impact of hydrolysate-associated inhibitors on major hydrolytic enzymes, a broad spectrum of inhibitor compounds present in different pretreated hydrolysates were investigated for the inhibition or deactivation of cellulases. These effects were evaluated on partially purified recombinant enzymes expressed in *Saccharomyces cerevisiae* (Table 1). The recombinant cellulases produced as monocomponent enzymes included the *Saccharomycopsis fibuligera* β-glucosidase 1 (SfCel3A), *Talaromyces emersonii* cellobiohydrolase 1 (TeCel7A) with a carbohydrate binding module (CBM) attached to its carboxyl terminal (TeCel7A-TrCBM), and *T. reesei* endoglucanase 2 (TrCel5A).

Table 3.1: Yeast strains used in this study

Strain	Genotype	Reference
Saccharomyces cerevisiae Y294	*a leu2-3,112 ura3-52 his3 trp1-289*	(den Haan et al. 2007)
Sc[Tecbh1-TrCBM-C]		(Ilmen et al. 2011)
Sc[*fur1::LEU2* ySF1]	*bla ura3/URA3 PGK_P-XYN SEC-BGL1-PGK_T*	(van Rooyen et al. 2005)
Y294 [TrCel5A]		(Brevnova et al. 2013)

3.2.1 Yeast strains, media and cultivation conditions

All chemicals, media components and supplements used in this study were of analytical grade and sourced from Sigma-Aldrich (South Africa). The yeast strains were maintained at 30ºC on YPD agar plates. Strains were cultivated in 2 L Erlenmeyer flasks in 500 mL double strength SC media (3.4 g/L yeast nitrogen base w/o amino acids and ammonium sulphate, 10 g/L ammonium sulphate, 20 g/L glucose and supplemented with amino acids as required), buffered to pH 6 with 0.17 M succinate buffer. Single colonies from 3-day old plates were used to inoculate the liquid cultures.

3.2.2 Partial purification of enzymes

Partial purification of TeCel7A-TrCBM, TrCel5A and SfCel3A was carried out as reported by den Haan et al. (2013). After 4 days of cultivation in SC medium at 30°C with orbital shaking at 200 rpm, cultures were centrifuged and the supernatant filtered through 0.45 µm glass

fibre filters (Millipore, Molsheim, France). The filtrate was subjected to the Minitan system (Millipore, Molshein, France) with a stacked ultrafiltration membrane with a 5 kDa cut-off to concentrate the samples to 200 mL, followed by the Amicon ultrafiltration system (Millipore, Molsheim, France) fitted with a 30 kDa cut-off membrane to concentrate the samples to 50 mL. The sample was dialyzed with two volumes of 50 mM Na-acetate buffer (pH 5.0) to a final volume of 50 mL. Protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules).

3.2.3 Hydrolysate inhibitor compounds

Selected compounds prevalent in lignocellulosic hydrolysates were added at concentration within the ranges reported in literature (Martin et al. 2003, Klinke et al. 2004). All the reagents were sourced from Sigma-Aldrich (South Africa) unless stated otherwise. These compounds included aliphatic acids (acetic acid and formic acid) at a concentration of 75 mM; furans (5-furfural and HMF) at 30 mM; and phenolic compounds (4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, 4-hydroxyacetophenone, syringaldehyde, vanillin, 4-hydroxy-3-methoxy-cinnamaldehyde (coniferyl aldehyde), cinnamic acid) at 1 mM. Tannic acid was also included as a phenolic compound at 1 mM to represent complex lignin. The inhibitor compounds were evaluated at 25%, 50%, 75% or 100% of the concentrations indicated above. All compounds were dissolved in distilled water, except cinnamic acid that was dissolved in 1.3 M methanol. The inhibitor compounds were filtered with a 0.2 µm Acrodisc® 25 mm PF syringe filter (Pall Corporation, USA) and stored at 4°C until use.

3.2.4 Enzyme activity assays

Cellulase and β-glucosidase activities were measured as described by the International Union of Pure and Applied Chemistry (IUPAC). The inhibition or deactivation effects were quantified by incubating the enzyme, inhibitor and substrate and measuring the enzyme activities on the appropriate substrate immediately (inhibition) or after 1, 6 and 24 hours (deactivation) (Ximenes et al. 2011). For β-glucosidase activity, enzyme preparations were incubated for 2 minutes at 50°C in 50 mM sodium acetate buffer (pH 5.0) with *p*-nitrophenyl glucopyranoside (pNPG) in the presence of varying concentrations of individual inhibitors and enzyme activity was expressed as previously described (den Haan et al. 2007). Inhibitor compounds such as HMF, coniferyl aldehyde, syringaldehyde and tannic acid that produced background colour with pNPG, were assayed with 15 mM cellobiose as substrate; treatment was carried out for 10 minutes in 50 mM sodium acetate buffer (pH 5.0) and the glucose

released was quantified using a D-glucose assay kit (GOPOD format) supplied by Megazyme (Wicklow, Ireland).

Quantification of TeCel7A-TrCBM activity was similar to that for SfCel3A, except that the assay was carried out for 15 minutes with *p*-nitrophenyl cellobiose (pNPC) as substrate. Inhibitor compounds such as coniferyl aldehyde, syringaldehyde and tannic acid that produced background colour with pNPC were assayed using soluble 4-methylumbelliferyl-β-D-lactopyranoside (MULac) as substrate (Ilmen et al. 2011). The reaction was terminated by increasing the pH with Na_2CO_3. For MULac assays, activities were determined using a fluorimeter (excitation 355 nm and emission 460 nm).

Endoglucanase activity was measured immediately after incubation of enzyme, carboxyl methyl cellulose (CMC) substrate with individual inhibitors at varying concentrations using a modified microtitre dinitrosalicylic acid (DNS) method (den Haan et al. 2007). Briefly, the reaction was stopped after 30 minutes by adding DNS and heated at 99 ºC for 5 minutes. The endoglucanase activity was determined spectrophotometrically at 540 nm, with one unit of cellulase activity (CMCase) defined as the release of one micromole of glucose equivalent per minute from CMC. No alternative substrates were available for HMF, coniferyl aldehyde, syringaldehyde and tannic acid. Hydrolysis experiment results were reported as an average of three independent assays with the standard deviation indicated.

3.2.5 Avicel hydrolysis

Selected compounds representing lignocellulosic hydrolysate inhibitors were incubated with 1% Avicel and a mixture of TeCel7A-TrCBM: TrCel5A at a protein concentration ratio of 9:1 (final loading of 50 mg protein/g glucan). Novozyme 188 (30 µL) was added as a source of BGL to prevent accumulation of glucose and feedback-inhibition of exo- and endo-cellulases. The reaction mixture was incubated at 35°C on a microtitre plate shaker for 48 hours at 1000 rpm and samples were taken at 24 and 48 hours. A modified DNS method was followed as described above to quantify sugars released (den Haan et al. 2007).

3.3 RESULTS

3.3.1 Inhibition/ deactivation effects on β-Glucosidase 1

The effect of various inhibitors generally present in lignocellulosic hydrolysates on SfCel3A was quantified by measuring the remaining hydrolytic enzyme activity immediately after exposing the enzyme to each inhibitor compound. Formic acid (Fig. 3.1a) and tannic acid (Fig.3.1c) at a concentration of 18.75 mM and 0.25 mM, respectively, demonstrated a strong inhibitory effect on BGL1. When the concentration of these two compounds was increased to 100% (i.e. 75 mM and 1 mM, respectively), a complete loss of β-glucosidase activity was evident. A drastic decrease in β-glucosidase activity was also observed with 75 mM acetic acid. Most of the other compounds had a negligible negative effect on SfCel3A activity, with some enhancing SfCel3A activity. The levels of residual SfCEL3A activity retained at 50% and 75% inhibitor concentrations are provided in Tables 1 and 2 of Additional file 1.

After incubating the enzyme-inhibitor complex for 24 hours (Fig. 3.1b and 3.1d), the enzymatic activities in the presence of most compounds remained within the same range as for 1 hour incubation as shown in Table 3 of Additional file 1. This suggested that for most compounds, the inhibition of SfCel3A was due to the presence of the specific inhibitor compounds rather than the contact time between enzyme and inhibitor. Furthermore, it could suggest that the inhibitor concentration was in excess on the enzyme surface which could have resulted in the observed inhibition. The strong inhibition observed for acetic, formic and tannic acid was therefore due to immediate inhibition as no significant changes in enzyme activity were evident after 24 hours of incubation (Fig. 3.1b and 3.1d). Increasing concentrations of coniferyl aldehyde and syringaldehyde resulted in a significant decrease in SfCel3A activity (Fig. 3.1d), which was more profound after 24 hours. Also refer to Tables 3 and 4 (Additional file 1) for residual SfCel3A activities at 1 and 6 hour intervals.

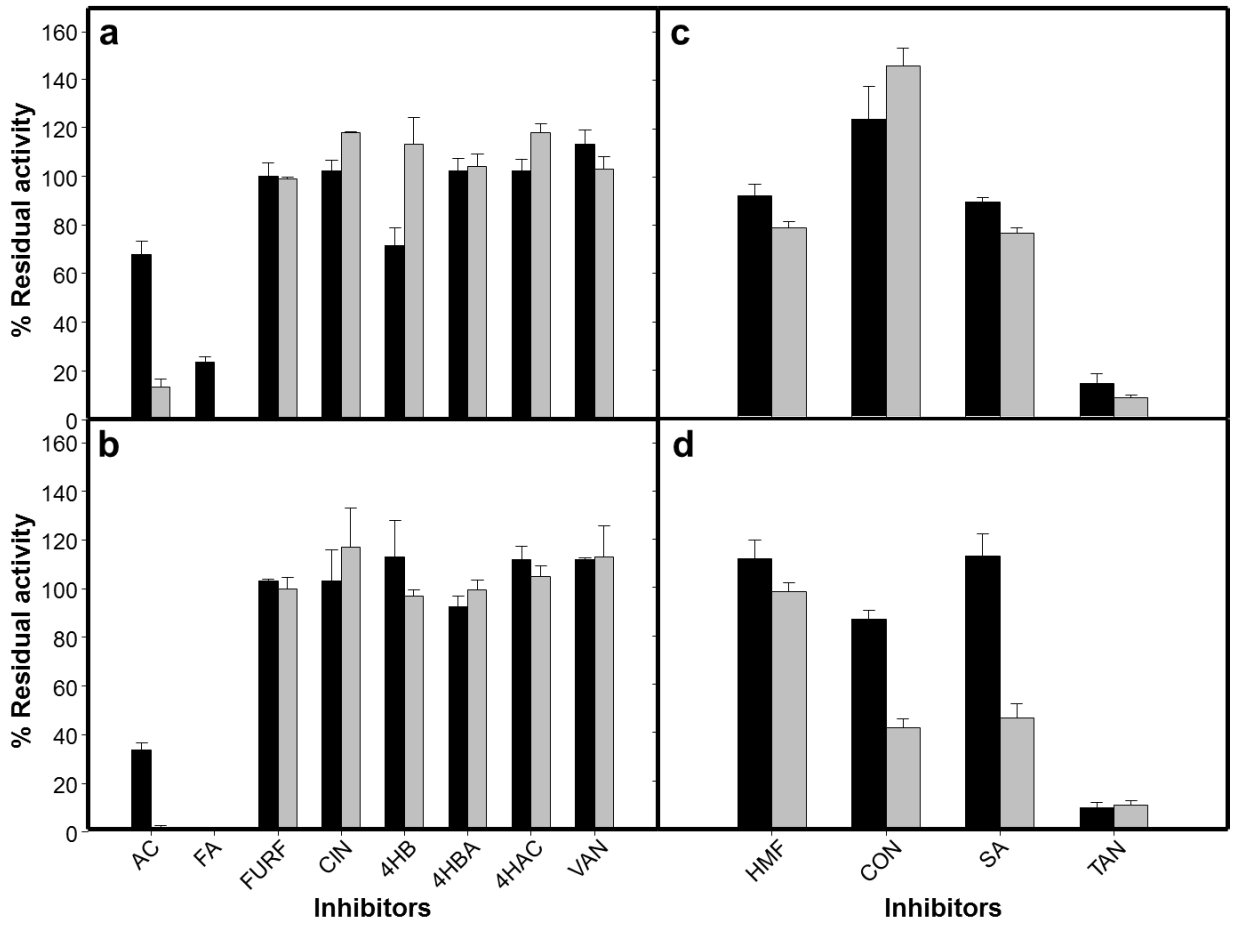

Figure 3.1 Inhibitory effects of lignocellulose associated model compounds on SfCel3A activity with (a) pNPG and (b) Cellobiose substrates and 24 hours of incubation deactivation effects on (c) pNPG and (d) cellobiose substrates. 100% concentration of inhibitors refers to 75 mM acetic acid (AC) and formic acid (FA), 30 mM furfural (FURF) and HMF and 1.0 mM cinnamic acid (CIN), 4-hydroxybenzaldehyde (4-HB), 4-hydroxybenzoic acid (4-HBA), 4-hydroxyacetophenone (4-HAC), vanillin (VAN), syringaldehyde (SA), coniferyl aldehyde (CON) and Tannic acid (TAN). Activities measured in the absence of inhibitors were used as reference for calculation of residual enzyme activity after inhibition. Results shown represent the mean value of three repeats and standard deviation is indicated with error bars. Black and grey filled bars represent 25% and 100% inhibitor concentrations, respectively.

3.3.2 Inhibition/deactivation effects on Cellobiohydrolase 1

A strong inhibition of TeCel7A-TrCBM activity was observed with 1.0 mM cinnamic acid (Fig. 3.2a), tannic acid, coniferyl aldehyde and syringaldehyde (Fig. 3.2c). Tannic acid completely shut down TeCel7A-TrCBM activity, whereas formic acid, furfural, hydroxymethylfurfural (HMF), hydroxybenzaldehyde and vanillin all showed a substantial inhibitory effect after exposure to either 25% or 100% of the inhibitor concentrations. A drastic drop of approximately 53% in CBH1 activity was observed when the concentration of acetic acid was increased from 18.75 mM to 75 mM, whereas increasing the concentration of coniferyl

aldehyde and syringaldehyde from 0.25 mM to 1.0 mM, resulted in a 3-fold decrease in TeCel7A-TrCBM activity. The residual TeCel7A-TrCBM activities at 50% and 75% inhibitor concentrations are provided in Tables 5 and 6 of Additional file 1.

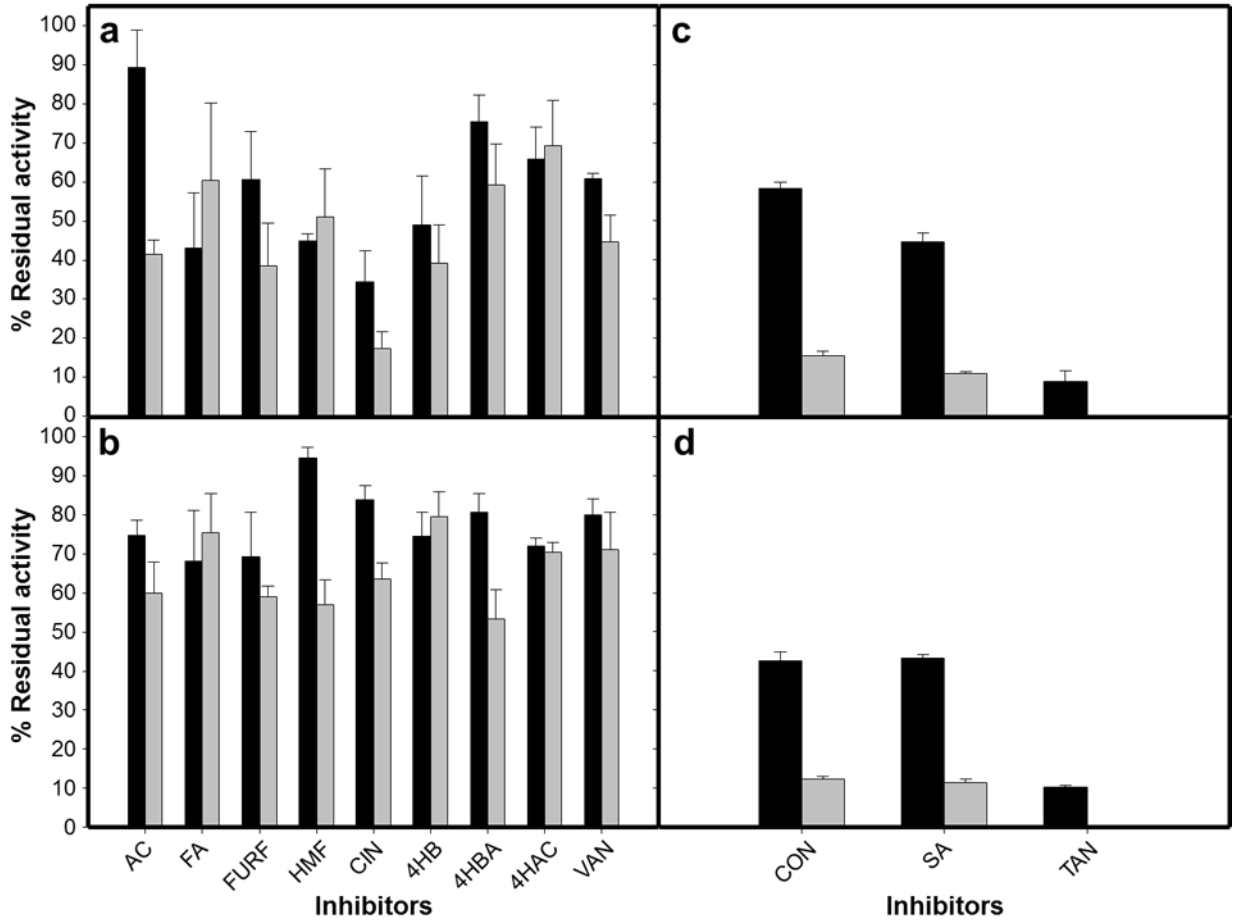

Figure 3.2 Inhibitory effects of lignocellulose-associated compounds on TeCel7A-TrCBM activity with (a) pNPC and (c) MULac substrates immediately after exposure, and deactivation effects on (b) pNPC and (d) MULac substrates after 24 hours of incubation. The concentrations of inhibitors are as stated in Figure 3.1. Activities measured in the absence of inhibitors were used as reference for calculation of residual enzyme activity after inhibition. Results shown represent the mean value of three repeats and error bars indicate standard deviations. Black and grey filled bars represent 25% and 100% of the inhibitor concentrations, respectively.

Most inhibitor compounds had a moderate inhibition effect on TeCel7A-TrCBM activity after 24 hours of incubation even at 25% of the inhibitor concentrations, whilst 100% HMF, cinnamic acid and 4-hydroxybenzoic acid substantially decreased TeCel7A-TrCBM activity (Fig. 3.2b). For example, TeCel7A-TrCBM activity decreased by approximately 58% in the presence of 25% coniferyl aldehyde and syringaldehyde, with an additional 3-fold decrease when 100% of these inhibitors was used. Tannic acid strongly inhibited TeCel7A-TrCBM at 25% and the enzyme completely lost its activity at 100% concentration of the inhibitor (Fig.

3.2d). The decrease in enzyme activity was therefore concentration-dependent rather than time-dependent. Also refer to Tables 7 and 8 (Additional file 1) for residual TeCel7A-TrCBM activities at 1 and 6 hour intervals.

3.3.3 Inhibition/deactivation effects on Endoglucanase 2

An approximate 50% decrease in TrCel5A activity was observed with 7.5 mM HMF, while 30 mM HMF resulted in complete inhibition (Fig. 3.3a). This was followed by tannic acid that strongly inhibited TrCel5A activity with a 74% decrease in activity at 1.0 mM. With the exception of vanillin and syringaldehyde, all the other compounds had a strong inhibition effect on TrCel5A activity, i.e. a loss of more than 50% activity at 100% of the respective concentrations. The residual TrCel5A activities at 50% and 75% inhibitor concentrations are provided in Table 9 of Additional file 1.

Different compounds reacted differently in response to increased inhibitor concentrations (Fig. 3.3b), but there was no evident time-dependent inhibition of TrCel5A. Increasing the concentration of cinnamic acid and acetic acid or prolonged incubation in the presence of these compounds had no effect on the enzyme activity. In the presence of coniferyl aldehyde and tannic acid, the activity decreased with increasing inhibitor concentration. However, an increase in the incubation time and concentration of the other compounds had a positive effect on TrCel5A activity. Also refer to Table 10 (Additional file 1) for residual TrCel5A activities at 1 and 6 hour intervals.

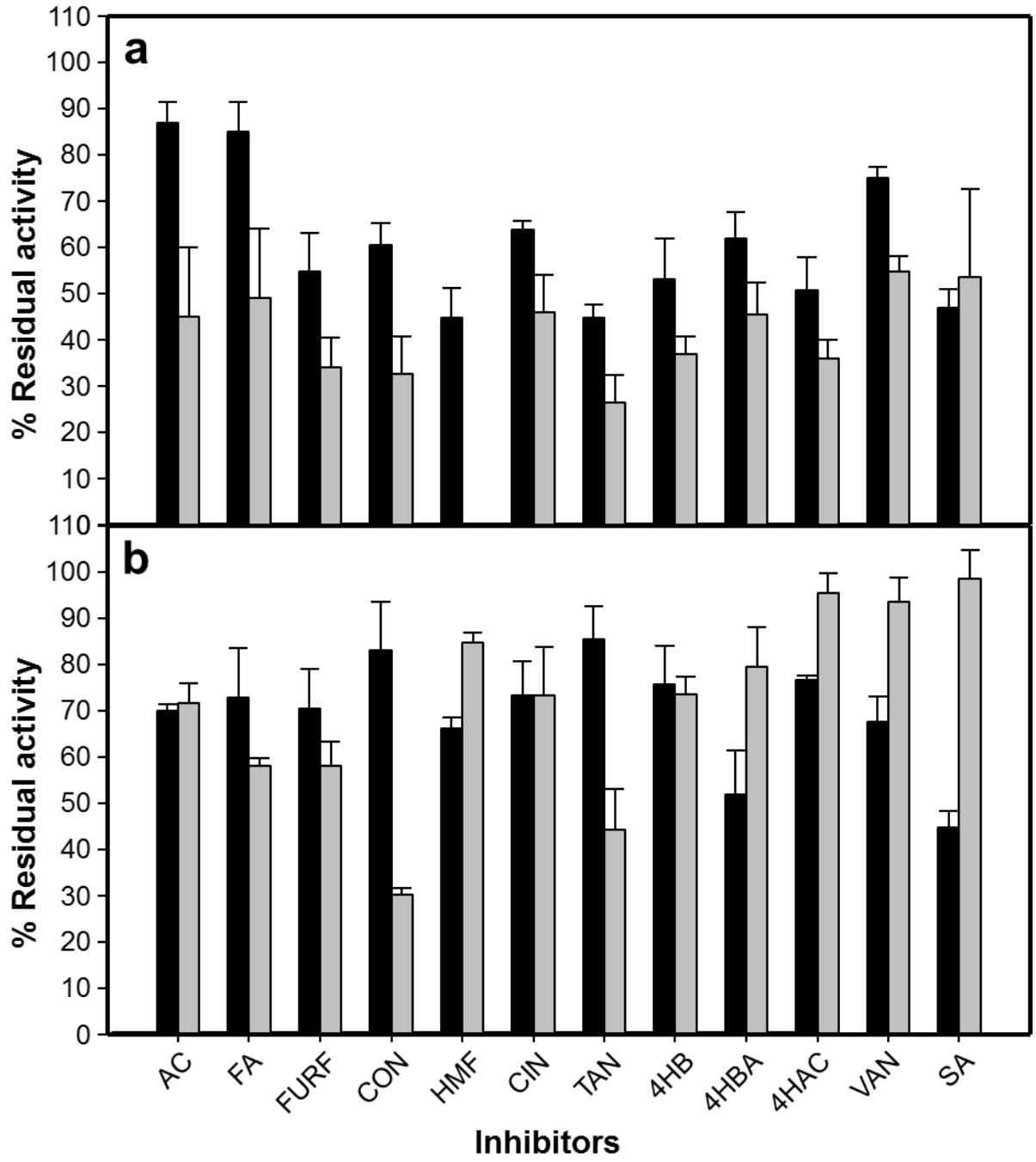

Figure 3.3 Inhibitory effects of lignocellulose-associated compounds on TrCel5A activity in terms of (a) immediate inhibition and (b) deactivation after 24 hour incubation period with CMC substrate. The concentrations of inhibitors are as stated in Figure 3.1. Activities measured in the absence of inhibitors were used as reference for calculation of residual enzyme activity after inhibition. Results shown represent the mean value of three repeats and error bars indicate standard deviations. Black and grey filled bars represent 25% and 100% of the inhibitor concentrations, respectively.

3.3.4 Avicel hydrolysis

The effect of inhibitors on the hydrolysis of Avicel by a mixture of TeCel7A-TrCBM, TrCEL5A and a commercial β-glucosidase was measured. In the absence of inhibitors, 7.2 g/L and 7.6 g/L Avicel was hydrolysed at 24 and 48 hours, respectively. The results are reported as percentage Avicel hydrolysed after 24 and 48 hours relative to the untreated control (Fig. 3.4). High enzyme loadings were used to clearly identify the inhibition effect on the combination of enzymes used. Acetic, formic and tannic acid substantially inhibited Avicel hydrolysis by TeCel7A-TrCBM:TrCel5A, with a decrease ranging from 46% to 58%. There were no significant differences in Avicel hydrolysis between 24 and 48 hours of treatment.

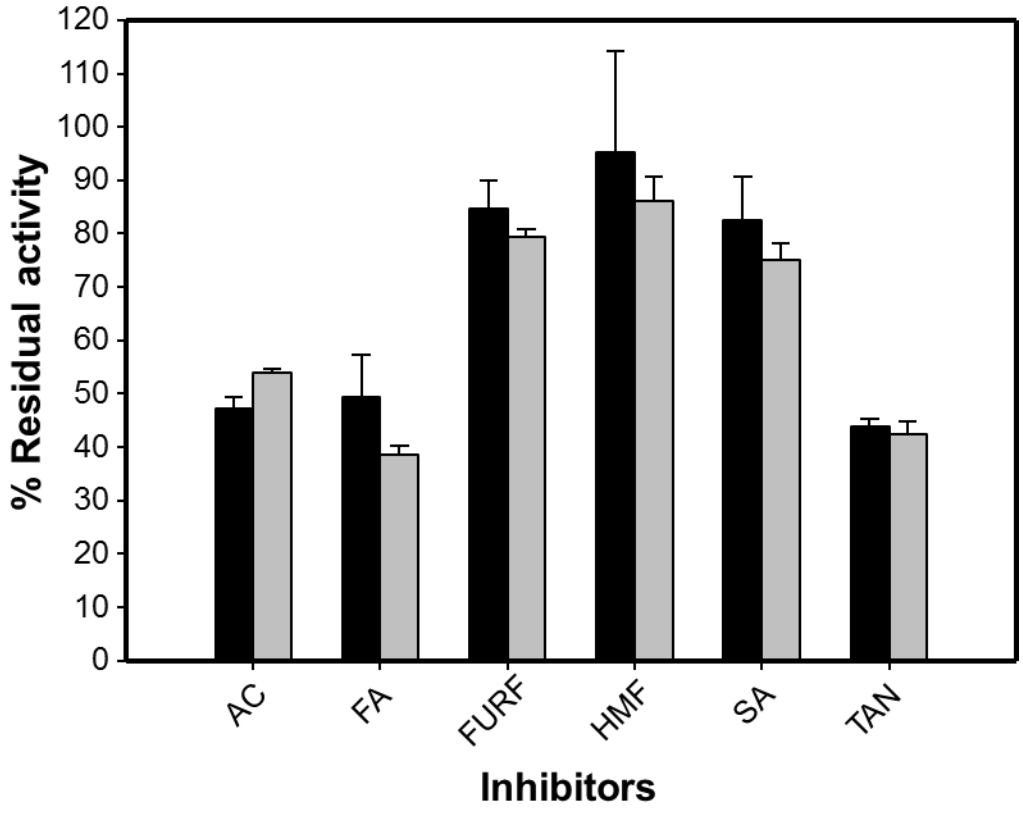

Figure 3.4 Inhibition effects of lignocellulose-associated compounds on the hydrolysis of 1% Avicel. Enzymes were applied at 50 mg protein/g glucan with TeCel7A-TrCBM: TrCel5A in a protein ratio of 9:1. Selected inhibitory compounds were added at a 100% concentration corresponding to 75 mM acetic acid (AC) and formic acid (FA); 30 mM furfural (FURF) and HMF; and 1.0 mM syringaldehyde (SA) and tannic acid (TAN). Results shown represent the mean value of three repeats and error bars indicate standard deviations. Black and grey filled bars represent 25% and 100% of the inhibitor concentrations, respectively.

3.4 DISCUSSION

Our results showed that the strong inhibition of enzyme activity was mostly due to the presence of polymeric lignin residues such as tannic acid and to a lesser extent by monomeric lignin compounds. Tannic acid strongly inhibited SfCel3A and TeCel7A-TrCBM, but had a lesser effect on TrCel5A. This is in agreement with previous reports by Ximenes et al. (2010 and 2011) that tannic acid is a strong inhibitor in comparison to other phenolic compounds. Furthermore, Ximenes et al. (2010) reported that the effect exerted by tannic acid was largely dependent on the source of enzyme, since the *Aspergillus niger* β-glucosidase (AnCel3A) was more resistant to inhibition than the *T. reesei* β-glucosidase (TrCel3A), which almost completely lost activity.

Increased concentrations of monomeric phenols such as cinnamic acid, coniferyl aldehyde and syringaldehyde showed strong inhibitory effects on TeCel7A-TrCBM activity. The pH of the reaction medium at 25% and 100% of the cinnamic acid concentration was 4.98 and 5.01, respectively, suggesting that the inhibition caused by cinnamic acid was due to the physical interaction between TeCel7A-TrCBM and cinnamic acid, rather than differences in pH.

Inhibition by compounds with an aldehyde functional group highlighted that different functional groups play a role in the inhibition effect of these compounds, but at different sensitivity levels. Eriksson et al (2002) indicated that surface properties such as hydrophobicity or hydrophilicity determine the level of toxicity for a specific inhibitor compound. Our results showed that hydrophilic compounds, such as hydroxybenzoic acid, did not significantly inhibit any of the cellulases investigated. However, the more hydrophobic compounds, such as tannic acid, had damaging effects. Polymeric phenols are well known to form complexes with enzymes and precipitate them from the solution (Kim et al. 2011). Endoglucanase 2 activity was inhibited most by HMF, with other inhibitor compounds showing moderate inhibition. Guo and co-workers (2014) showed that lignin preparations from corn stover and pine greatly adsorbed cellobiohydrolase and xylanase, while endoglucanase activity was less affected. The inhibitory effect of HMF on TrCel5A requires further investigation since HMF is produced as high levels in acid pretreated hydrolysates. Endoglucanse activity was observed to increase after a 24 hour incubation period for most phenolic compounds compared to activity at 1 hour incubation (see additional file). Endoglucanase is non-processive and has a grooved shaped catalytic site which allows it to randomly cleave cellulose chains (Zhang and Zhang 2013). We can speculate that the non-

processive nature of the endoglucanase active site allows this enzyme to escape non-productive binding due to hydrophobic interactions with phenolic inhibitor compounds through the same mechanism as in cellulose hydrolysis where it randomly nicks cellulose chains.

In addition to strong inhibition by tannic acid, weak acids also displayed strong inhibition of the cellulases: TeCel7A-TrCBM was strongly inhibited by formic acid, while SfCel3A was strongly inhibited by both acetic and formic acid. Acetic acid only showed strong inhibition towards TeCel7A-TrCBM activity when its concentration was increased to 100%. In control experiments without inhibitor compounds, the pH of the assay mixture remained at pH 5.2, but dropped to 4.9 or 4.63 when acetic or formic acid was added at 25%, and to 4.37 or 3.69 in the presence of 100% of the inhibitors, respectively. The loss of SfCel3A activity upon the addition of weak acids and of TeCel7A-TrCBM activity with increased acetic acid concentrations can partially be attributed to the drop in pH which has been shown to change the surface charges of the enzyme and enhance non-specific binding (Rahikainen et al. 2013).

Since weak acids were only observed to have a strong inhibition effect on the retaining enzymes (SfCel3A and TeCel7A-TrCBM) and not on the inverting enzyme (TrCel5A), this could suggest that weak acids affect the double displacement retaining mechanism in glycoside hydrolases. Addition of exogenous nucleophile molecules (azide, acetate or formate) has been reported to directly attack the anomeric carbon centre thus substituting the nucleophile which resulted in the formation of an adduct molecule with an inverted configuration (Vuong and Wilson 2010, Naumoff 2011). This can suggest that the weak acid (acetic or formic acid) replaced the nucleophile in the retaining mechanism and formed a reversion product such as glycosyl-acetate (or formate). Further investigation on the observed specificity of weak acids on retaining enzymes will allow better understanding of the mechanism.

The time of exposure did not have a significant effect on cellulase activity, but a higher concentration of most inhibitor compounds significantly decreased the activity of the enzymes. This suggested that decreased hydrolysis rates may be due to other factors, such as substrate crystallinity or a combined effect of different inhibitor compounds present in a lignocellulosic hydrolysate. The ratio of inhibitors to lignin may also explain the observed inhibition since the inhibition was only decreased with increasing inhibitor concentration. It

has been shown that the inhibition can be mitigated by high enzyme loading which resulted in excess enzyme available to hydrolyse cellulose (Ko et al. 2015). Our results are in contrast with previous findings that increased contact time between lignin residues and cellulases lead to the deactivation of the enzyme (Ximenes et al. 2011, Ximenes et al. 2010, Kim et al. 2011), with a decrease of up to 60% in β-glucosidase activity over a 40-hour exposure to phenolic compounds reported (Kim et al. 2011). However, the latter involved a mixture of phenolic compounds that may act synergistically to deactivate the enzyme. In addition, the recombinant SfCel3A used in this study was overexpressed in *S. cerevisiae*, which may have altered its sensitivity when compared to commercial β-glucosidase used in other studies.

A combination of TeCel7A-TrCBM and TrCel5A with added commercial β-glucosidase Novozyme 188 was used to hydrolyse a microcrystalline lignocellulosic substrate (Avicel). Tannic, acetic and formic acid were the strongest inhibitors of Avicel hydrolysis by the enzyme combination, while other monomeric phenols showed less or no inhibition of Avicel hydrolysis over a 48 hour incubation period. Furthermore, monomeric phenols in particular only displayed a functional group-dependent inhibition at higher inhibitor concentrations. Ximenes et al. (2010) showed that monomeric lignin residues strongly inhibited cellulose hydrolysis, with a decreasing inhibition order from vanillin, syringaldehyde, trans-cinnamic acid to 4-hydroxybenzaldehyde. In the present study, only syringaldehyde was evaluated and did not show substantial inhibition.

Our results were obtained using high enzyme loading to clearly identify the inhibitory effects of added compounds on avicel hydrolysis within a 48 hour hydrolysis. Kim et al. (2011) showed that enzyme loading as low as 1 mg/mL could hydrolyse lignin-free cellulose and that addition of soluble inhibitors in preteated liquid resulted in approximately 50% decrease in 1% solka floc hydrolysis at 168 hours. However, there was only a 10% difference between the untreated and treated solka floc hydrolysis at 48 hours with low enzyme loadings while the difference was approximately 33% when high enzyme loading was used. This suggests that the hydrolysis time has to be extended to clearly distinguish the inhibition effect at low enzyme loadings. However, it is likely that a different pattern of inhibition could be observed at low enzyme loading.

3.5 CONCLUSION

The inhibition of cellulases largely depends on the specific relationship between the specific cellulase and inhibitor compound. In general, polymeric lignins (such as tannic acid) and weak acids (such as formic acid) resulted in severe inhibition of all three cellulases. The inhibition effect exerted by acetic acid and other monomeric phenols depended on the concentration of these compounds. This implies that pretreatment processes can be designed to limit the concentration of these inhibitor compounds or alternatively, enzymes can be engineered to enhance their tolerance for specific inhibitor compounds. Properties such as the hydrophobicity of both the enzyme and inhibitor compounds as well as functional groups on the surface of the inhibitor compounds could facilitate non-productive adsorption of the enzyme to phenolic inhibitor compounds. However, this also depends on the concentration of the specific inhibitor compound present in the hydrolysate. To our knowledge, the present study is the first report on the inhibition or deactivation effects of weak acids and furans on individual cellulases, unlike other studies that only focused on the effect of lignin and its residual compounds or direct lignocellulose hydrolysate containing a mixture of compounds which make it difficult to identify the compounds involved in inhibition. Furthermore, this study provides insight into the impact of individual inhibitors on hydrolytic enzymes, which in future will inform the appropriate pretreatment strategies for different substrates.

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Chapter 4

RESEARCH RESULTS II

Enhancing Enzymatic Hydrolysis of Cellulolytic Biomass by Applying Detoxification Strategies

CHAPTER 4:

Enhancing enzymatic hydrolysis of cellulolytic biomass by applying detoxification strategies

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Abstract

Lignocellulosic biomass is increasingly attracting attention as a potential future source of transportation energy. However, the recalcitrance of cellulosic feedstocks requires a pretreatment step to render the sugar polymers accessible to enzymatic hydrolysis. The release of inhibitory compounds during pretreatment limits the action of hydrolytic enzymes on the cellulosic substrate. Recently, sulfur oxyanions have been shown to significantly improve enzymatic hydrolysis and microbial fermentation of biomass to ethanol. This study investigated the impact of sulfur oxyanions, laccase and lignosufonate on the enzymatic hydrolysis of cellulose by recombinant cellulases using model inhibitor compounds and/or bagasse solids and pretreatment liquor as a source of inhibitors. The results showed that although the detoxifying agents reacted with individual inhibitors, none of the detoxifying agents significantly enhanced glucose production from Avicel. However, a substantial improvement was observed in the hydrolysis reaction of Avicel spiked with bagasse pretreatment liquid. In particular, 10 mM sodium dithionite improved Avicel hydrolysis by 28% relative to the control reaction (containing pure cellulose and citrate buffer). The study confirmed that detoxifying agents reacted with inhibitor compounds containing an aldehyde functional group, but this reaction did not enhance the cellulase performance. Furthermore, the detoxifying effect was more pronounced in reactions containing the pretreatment liquid than in the reactions containing the solid filter cake, suggesting that the detoxification was more effective on soluble inhibitors than insoluble inhibitors.

Keywords: Detoxification, Sodium dithionite, Sodium sulfite, Lignosulfonate, Laccase

4.1 INTRODUCTION

Lignocellulosic biomass offers a versatile option for the production of bioethanol and other fine chemicals (Soudham et al. 2014). However, the cost-effective production of cellulosic bioethanol is currently hindered by a number of techno-economic challenges, including a requirement for pretreatment to disrupt its recalcitrant structure prior to enzymatic hydrolysis. The most promising strategy for industrial application is steam pretreatment, where high temperatures and pressure are typically applied in combination with an acidic or basic catalyst to facilitate the disruption of the lignocellulosic polymers (Jönsson and Martin 2016). While the main purpose of pretreatment is to open up biomass fibres for enzymatic hydrolysis, it inevitably results in the release of by-products such as aliphatic acids, furans and phenolic compounds. These compounds are inhibitory to both the enzymatic hydrolysis and microbial fermentation steps (Almeida et al. 2007, Kim et al. 2015).

In addition to inhibition by soluble pretreatment by-products, lignin also interferes with enzymatic hydrolysis by adsorbing cellulases that results in the precipitation, inhibition and/or deactivation of the cellulases (Kim et al. 2011, Tejirian and Xu 2011). Lignin is a hydrophobic aromatic plant cell wall polymer primarily composed of three precursors, *ρ*-coumaryl, coniferyl and sinapyl alcohol. The diversity and distribution of phenolic structures in the lignin polymer determine the magnitude of its impact on enzymatic hydrolysis (Zheng et al. 2013, Nakagame et al. 2010). The mechanism of inhibition by lignin and its derivatives is poorly understood, but a number of studies have reported on its inhibition/deactivation effects (Ximenes et al. 2011, Kim et al. 2011, Mhlongo et al. 2015). The lignin-cellulase interaction during enzymatic hydrolysis of lignocellulosic biomass can occur through two mechanisms that can be described as (i) blockage of cellulase access to cellulosic substrate due to steric hindrance by lignin, and (ii) non-productive adsorption of cellulases onto lignin. Both these lignin-cellulase interactions result in inefficient enzymatic hydrolysis and low sugar yields (Wang et al. 2013).

Lignin is not removed significantly during the pretreatment process, but rather partially solubilised and relocated, which improves the access of cellulases to the cellulosic material (Zheng et al. 2013). Complete delignification might improve enzymatic hydrolysis, but is considered too expensive on an industrial scale and can result in the degradation of fermentable sugar polymers due to harsher treatment conditions (Wang et al. 2013, Varnai et al. 2010). Removal of hemicellulose also improves the enzymatic hydrolysis of cellulosic biomass, possibly due to decreased structural complexity and crystallinity as a result of

increased pore volume and specific surface area (Varnai et al. 2010). Pretreatment strategies such as steam explosion introduce chemical changes that enhance the affinity of lignin for cellulases. Phenolic hydroxyl groups on the surface of lignin residues increase the surface hydrophobicity of lignin and thus the level of toxicity, while carboxylic acid groups increases lignin hydrophilicity that alleviates enzyme inhibition (Nakagame et al. 2011, Zheng et al. 2013, Rahikainen et al. 2013).

A number of strategies have been proposed and investigated to circumvent inhibition problems (Jönsson and Martin 2016). These strategies include the optimisation of pretreatment conditions or the design of fermentation conditions to minimise inhibitory compounds, the selection, adaptation or engineering microbial catalysts for improved resistance towards inhibitors and/or engineering cellulases for decreased affinity for non-productive binding to inhibitors (Alriksson et al. 2011). The manipulation or selection of pretreatment and process conditions to minimise the release of inhibitors can have a negative impact on sugar yield as well as the ethanol yield and productivity. Engineering robust cellulases or microbial catalysts is an interesting approach, but this is limited to lignocellulose hydrolysates with a lower inhibitory potential (Cavka et al. 2011). Lignocellulose hydrolysate inhibitors can also be removed with a wash step that removes inhibitors along with water-soluble hemicelluloses absorbed in the solid filter cake. The wash step is not commercially applicable since it also removes fermentable sugars and generates highly diluted final products that require subsequent distillation for recovery (Sun and Cheng 2002). The washing step is also water-intensive since it requires about 10 m^3 water/ton substrate and disposal of the wastewater raises environmental concerns (Wang et al. 2013). Although recirculation of process water can save cost and minimise the environmental impact, it can also further concentrate the inhibitors (Alriksson et al. 2011).

Another interesting approach is to use surfactants and/or non-catalytic proteins such as bovine serum albumin (BSA) to reduce non-productive binding of the cellulases (Alriksson et al. 2011). Addition of polyethylene glycol (PEG) and Tween increases biomass hydrolysis by competitively adsorbing to lignin, releasing cellulases from unproductive binding to lignin and possibly also enhancing the thermal stability of cellulase. The surfactants are proposed to occupy the hydrophobic sites on the surface of the inhibitor compound and thus displacing the adsorbed enzyme (Eriksson et al. 2002). The addition of surfactant can also increase the desorption rate of cellulases, thus increasing free enzyme for cellulose conversion (Hsieh et al. 2015). The drawback of this approach is the high cost of surfactant or enzymes required

for large-scale lignocellulosic biomass processing (Wang et al. 2013). In addition, surfactants such as Triton X contain an aromatic ring and can be harmful to the environment when used in large quantities (Eriksson et al. 2002).

Other detoxification strategies include chemical, physical and biological detoxification that could circumvent inhibition even in severely inhibitory hydrolysates (Mussatto and Roberto 2004). Detoxification has a great advantage over other proposed strategies since it can permit full process flexibility and application in a large-scale industrial process without the introduction of organisms genetically manipulated for high inhibitor tolerance. It is important to develop detoxification processes that selectively remove inhibitors, efficiently detoxify strongly inhibitory hydrolysates, and are inexpensive and compatible with other processing steps (Palmqvist and Hahn-Hagerdal 2000, Guo et al. 2014). The use of chemical *in situ* detoxification has recently been shown to be a promising tool to circumvent inhibition problems in the fermentation vessel without the introduction of a separate step (Alriksson et al. 2011). The aim of this study was to gain insights into the potential of detoxifying agents to enhance enzymatic hydrolysis of cellulosic substrates by recombinant cellulases, as well as the impact of selected chemical agents on specific inhibitory compound groups found in pretreated lignocellulosic substrates. We further compare the chemical detoxification to enzymatical detoxification by treatment of the reactions containing inhibitor compounds with a phenol oxidase laccase, which is an enzyme known to remove phenolic compounds (Martin et al. 2002).

4.2 MATERIALS AND METHODS

4.2.1 Substrates, chemicals and reagents

All chemicals were sourced from Sigma Aldrich (South Africa or Sweden) unless indicated otherwise. Avicel PH101 represented pure cellulose; model inhibitor compounds representing sugar degradation products and lignin-derived compounds included tannic acid, coniferyl aldehyde and syringaldehyde. Additives for *in situ* detoxification included sodium dithionite and sodium sulfite. Steam-explosion pretreated sugarcane bagasse was kindly provided by David Naron (Bioprocess Research group, Department of Process Engineering, Stellenbosch University). The pretreatment was performed in a 19-L reactor vessel after 1 kg bagasse was soaked in 10 L water for 24 hr. The water was removed by centrifugation and the material was treated with steam for 13.5 min at 205°C. The pretreated slurry was collected from the cyclone, cooled to room temperature and pressed with an industrial-scale

hydraulic press to 40% (w/w) moisture content. The pressed bagasse (filter cake) and pressed liquor were collected and stored at -20°C.

4.2.2 Chemical analysis of sugarcane bagasse

The steam-pretreated sugarcane bagasse was acid-hydrolysed as per the NREL method (Sluiter et al. 2008) and dried overnight at 30°C. Approximately 3 mg aliquots of the dried material were mixed with 3 mL of 72% sulphuric acid to a homogenous solution; the tubes were incubated at 30°C in a water bath for 1 hr with stirring every 15 min. The samples were then diluted with 84 mL of distilled water, transferred to 250 mL glass bottles and autoclaved at 121°C for 60 min. The samples were cooled to room temperature and filtered using a pre-weighed crucible. Duplicate 10-mL samples of the filtrate were stored at 4°C for soluble lignin and carbohydrate analysis. The crucibles containing solid lignin were placed in a 100°C incubator to dry for 24 hr.

Sugars, acetic acid and formic acid present in the acid-hydrolysed sugarcane bagasse were analysed on an Aminex HPX-87H column equipped with a Cation-H Micro-Guard cartridge (Bio-Rad, Johannesburg, South Africa) operating at 65°C with 5 mM sulphuric acid as mobile phase and a flow rate of 0.6 ml/min. Sugars, acetic acid and formic acid peaks were detected with an Shodex RI-101 detector operated at 45°C. The by-products HMF and furfural were quantified on a Luna C18 (2) reversed-phase column equipped with a Luna C18 (2) precolumn (Phenomenex) operating at 25°C and a flow rate of 0.7 ml/min. The mobile phase for elution was 5 mM trifluoroacetic acid in acetonitrile. Separation was achieved with gradient elution starting with 5% mobile phase, increasing to 11% mobile phase over 14 minutes and then increasing to 40% mobile phase over 3 minutes. The mobile phase composition was then kept constant at 40% for 2 minutes, followed by a decrease to 5% mobile phase over 5 minutes and ending with a final step of constant composition at 5% mobile phase for 4 minutes in order to equilibrate. HMF and furfural peaks were detected with a Dionex Ultimate 3000 diode array detector at 215 nm and 285 nm.

Phenolic compounds were analysed by HPLC on an XSelect HSS T3 reversed-phase column (4.6 x 250 mm, 3.5 µm particle size) equipped with an XSelect HSS T3 precolumn (Waters Corporation, Milford, MA, USA) operating at 37 °C and a flow rate of 0.7 ml/min. Separation was carried out by gradient elution with an initial isocratic step at 0% mobile

phase for 5 minutes, increasing to 15% mobile phase B during the next 25 minutes, then increasing to 35% mobile phase during the next 50 minutes and then increasing to 100% mobile phase during the next 5 minutes. The mobile phase composition was then kept constant at 100% mobile phase for 5 minutes, followed by a decrease to 0% mobile phase during the next 7 minutes and ending with a final step of constant composition at 0% mobile phase for 7 minutes in order to equilibrate. Phenolic compounds peaks were detected with a Dionex Ultimate 3000 diode array detector at 285 nm and quantification was done using external standard calibration curves.

4.2.3 Detoxification of bagasse and model compounds

The detoxification reactions with model compounds (coniferyl aldehyde, syringaldehyde and tannic acid) were performed in 10 ml Falcon tubes at 45°C with 10% Avicel as pure cellulosic substrate. All the hydrolysis experiments were carried out for 72 hr with shaking at 170 rpm and samples were taken at 24 hr intervals. The detoxification reactions contained reducing agents (10 mM sodium sulfite or dithionite) or 0.45 U/mL phenol oxidase laccase from *Trametes versicolor* (Fluka Biochemika, Switzerland) as detoxifying agents. Laccase activity was determined by oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-suphonic acid (ABTS) at 420 nm and the enzyme activity expressed in units, where one unit was the amount (µmol) of ABTS oxidised per min (Bourbonnais et al. 1998). Treatment of model compounds was initiated by mixing the model inhibitor with laccase at 45°C for 12 hr before the addition of 10% Avicel. Glucose concentrations were measured with a glucometer (Accu-Check Aviva, Roche Diagnostics, Basel, Switzerland) as described by Normark et al. (2016).

The detoxification experiments with bagasse filter cake and/or pretreatment liquor were performed in 100 mL conical flasks covered with parafilm and incubated at 37°C for 72 hrs. The reaction mixture contained 2% glucan (either pure Avicel spiked with pretreatment liquor from sugarcane bagasse, or pressed bagasse filter cake), with the control reaction containing pure Avicel (2%) in 50 mM citrate buffer. The pH was adjusted to pH 5.0 using 5 M NaOH. Either sodium sulfite or sodium dithionite were added at 10 mM and pre-incubated for 10 min prior to the addition of cellulases (sulfur oxyanions were omitted from control experiments). The extent of hydrolysis was measured using a modified DNS method (Den Haan et al. 2007).

The hydrolytic cellulases, TeCel7A-TrCBM and TrCel5A, were isolated and partially purified from recombinant yeast strains (Mhlongo et al. 2015). An enzyme load of 2% (with a TeCel7A-TrCBM to TrCel5A ratio of 7:3) was supplemented with 0.5% β-glucosidase (Novozyme 188, Sigma-Aldrich, St Louis, MO, USA) to facilitate cellobiose hydrolysis and to avoid feedback inhibition of cellobiohydrolases by the accumulation of cellobiose. Residual TeCel7A-TrCBM activity was quantified with a fluorescence-based method (Ilmen et al., 2011) using 4-methylumbelliferyl-β-D-lactoside (MULac, Carbosynth, UK) as substrate.

4.3 RESULTS

In the presence of coniferyl aldehyde, a significant inhibition of enzyme hydrolysis was observed after 72 hrs compared to non-inhibiting conditions in the control reaction (Fig 4.1a). The inhibition by coniferyl aldehyde was relieved with the addition of 10 mM sodium sulphite, whereas 10 mM sodium dithionite did not show a significant impact. Since TeCel7A-TrCBM was added in excess (70% of the cellulase load), it served as a good indicator of the impact of the inhibitors and/or detoxification on enzyme activity. After 72 hrs, only 62.5% residual TeCel7A-TrCBM activity was observed in the presence of coniferyl aldehyde, with slightly higher activity levels in the presence of either of the sulfonates (Fig 4.1b).

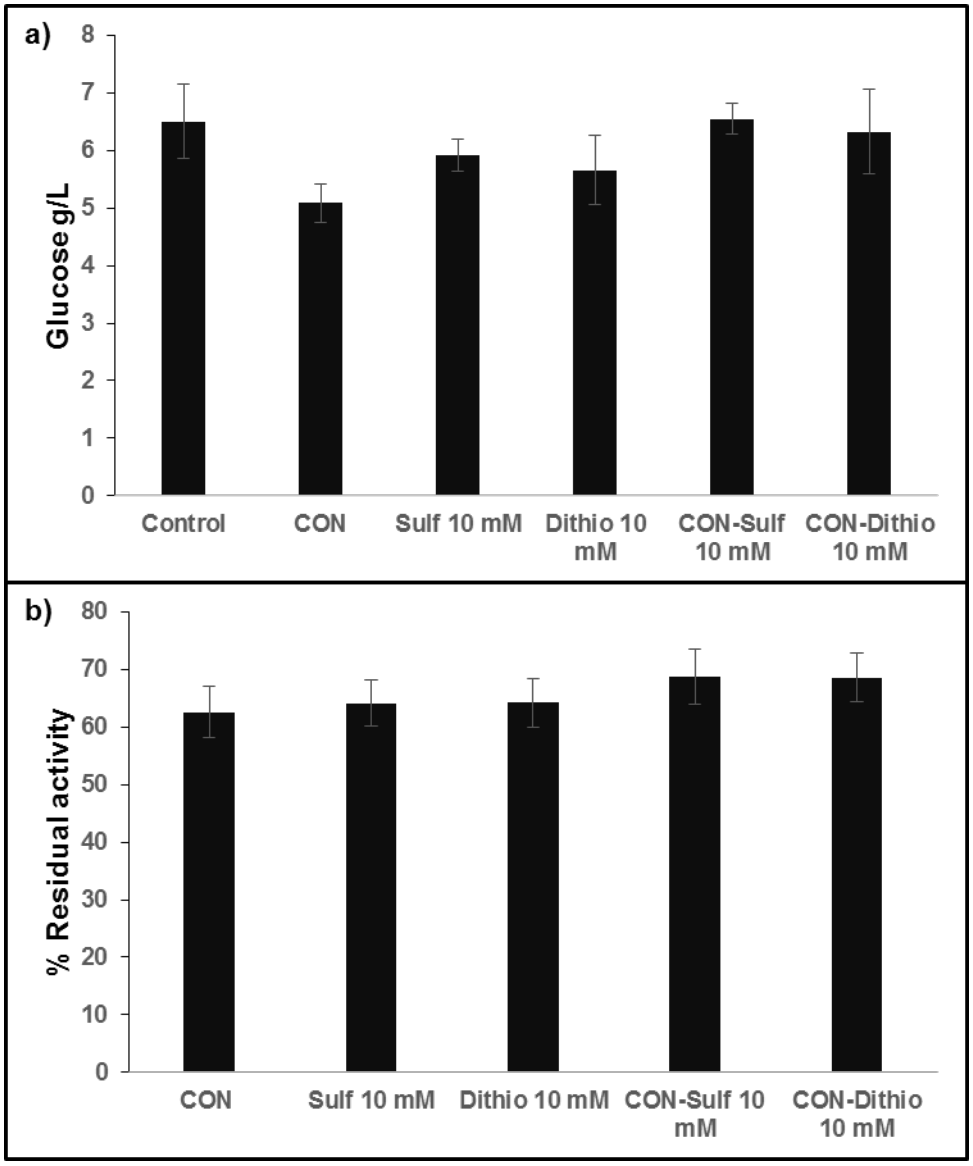

Figure 4.1 Impact of detoxification with 10 mM sodium sulfite (Sulf) or sodium dithionite (Dithio) on cellulase hydrolysis of 10% Avicel in the presence of coniferyl aldehyde (CON) after 72 hr. (a) Enzymatic hydrolysis as measured by glucose release. (b) Quantification of TeCel7A-TrCBM residual activity as percentage activity remaining in the reaction containing inhibitor only or detoxifying agent only or both after 72 hr with reference to the reaction containing no inhibitor or detoxifying agent. Results represent the mean value of three independent repeats and error bars indicate the standard deviation.

There was no clear inhibition of enzymatic hydrolysis in the presence of syringaldehyde sodium sulfite or dithionite (Fig.4. 2), but a decrease in glucose production was observed when sodium sulfite was added together with syringaldehyde. Interestingly, residual TeCel7A-TrCBM activity was reduced in reactions containing syringaldehyde although glucose production was not affected. The TeCel7A-TrCBM activity was decreased to 42.9%

in the presence of syringaldehyde, whereas the addition of 10 mM sodium sulfite and 10 mM sodium dithionite under inhibiting conditions partially restored TeCel7A-TrCBM activity to 58.6% and 50%, respectively,

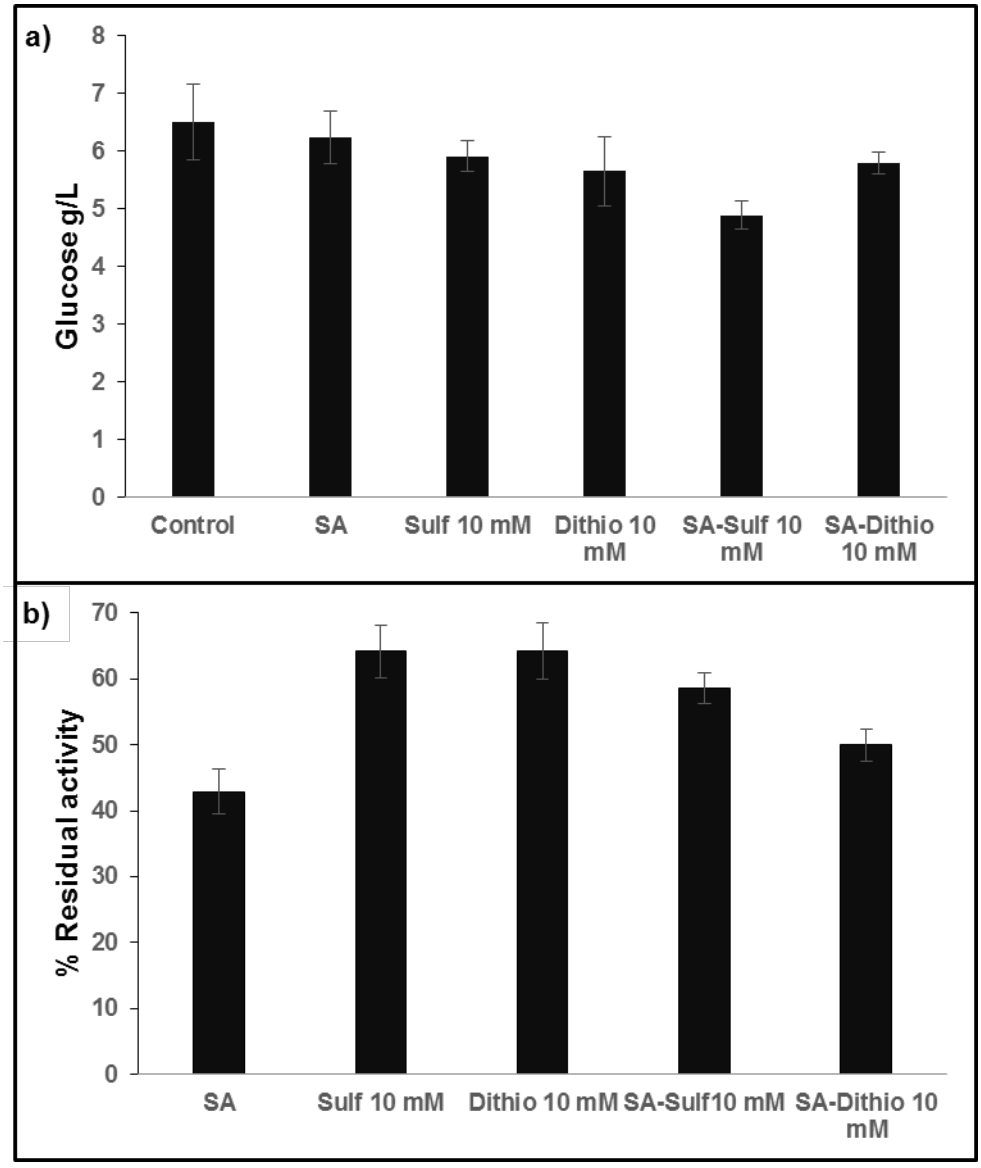

Figure 4.2 Impact of detoxification with 10 mM sodium sulfite (Sulf) or sodium dithionite (Dithio) on cellulase hydrolysis of 10% Avicel in the presence of 1 mM syringaldehyde (SA) after 72 hr. (a) Enzymatic hydrolysis as measured by glucose release. (b) Quantification of TeCel7A-TrCBM residual activity as percentage activity remaining in the reaction containing inhibitor only or detoxifying agent only or both after 72 hr with reference to the reaction containing no inhibitor or detoxifying agent. Results represent the mean value of three independent repeats and error bars indicate the standard deviation.

Coniferyl aldehyde significantly inhibited the release of glucose from Avicel (Fig. 4.3). Although the addition of laccase improved glucose production from 4.4 g/L to 5.1 g/L in the

reaction with coniferyl aldehyde, the inhibitory effect of coniferyl aldehyde was still evident. Tannic acid also reduced the glucose yield to 5.3 g/l, with no significant impact when laccase was added.

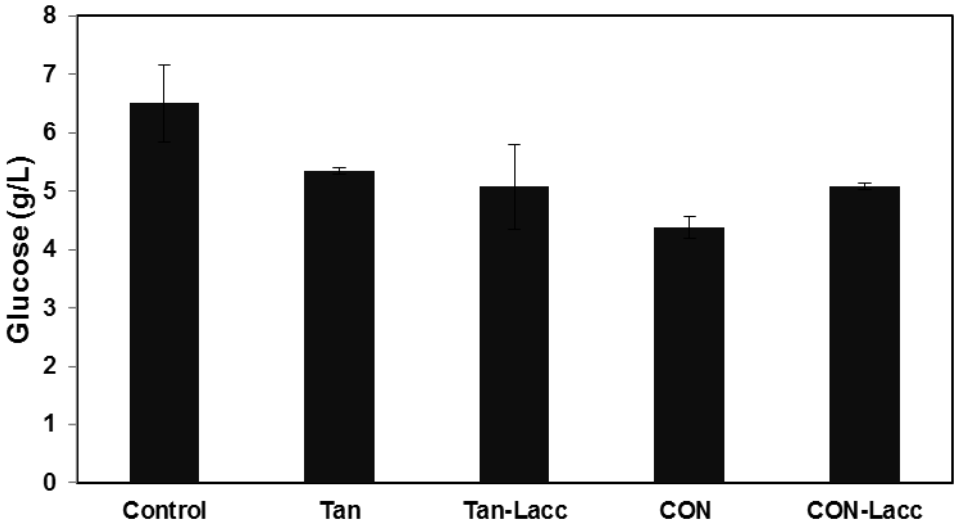

Figure 4.3 Impact of 0.45 U/mL laccase (Lacc) on glucose production from 10% Avicel hydrolysed by cellulases in the presence of 1 mM tannic acid (Tan) or coniferyl aldehyde (CON). The reaction lacking the inhibitor and detoxifying agent was included as a control reaction. Results represent the mean value of three independent repeats and error bars indicate the standard deviation.

The results presented in Fig. 4.1 to 4.3 indicated that the addition of sulfonating agents slightly reduced the hydrolysis of pure Avicel, suggesting that the detoxifying agents itself may inhibit enzymatic hydrolysis. To ascertain the effect of detoxification by sulfonating agents on a complex cellulosic substrate, experiments were conducted with the solid (filter cake) and liquid (hemicellulose liquor) fractions of bagasse. As shown in Fig. 4.4, the hydrolysis of Avicel was significantly reduced in the presence of the filter cake, independent of the presence of any of the sulfonates. The presence of 10% pretreatment liquid had a similar inhibitory effect on the enzymatic hydrolysis of Avicel, but this was relieved to some extent in the presence of the detoxifying agents: 10 mM lignosulfonate and sulfite improved Avicel hydrolysis by 4.1% and 8%, respectively, relative to the absence of detoxifying agents. An exceptional improvement of 28% in hydrolysis was observed in the presence of 10 mM sodium dithionite, which even superseded the hydrolysis of pure Avicel in the absence of sulfonates.

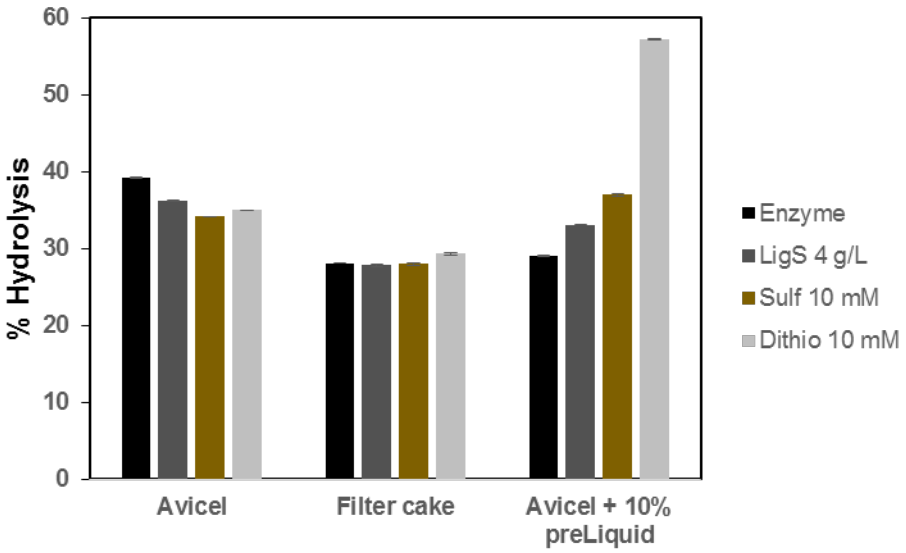

Figure 4.4 Detoxification of sugarcane bagasse (SCB) fractions (filter cake and/or hemicellulose liquor fractions). The reactions contained either 2% (w/w) Avicel, 2% (w/w) SCB filter cake or 2% (w/w) spiked with 10% (v/v) pretreatment liquor. Selected detoxifying agents included 10 mM sodium sulfite (Sulf) or sodium dithionite (Dithio) or 4g/L lignosulfonate (LigS) as indicated. The results are expressed as percentage cellulose converted with respect to the initially added cellulose in the reaction. Results represent the mean value of three independent repeats and error bars indicate the standard deviation.

Chemical analyses of the solid (filter cake) and liquid (pretreatment liquor) fractions following pretreatment, indicated that the bagasse filter cake was mainly composed of sugars (glucose) and lignin (Table 4.1). The liquor contained a diversified group of hemicellulose sugars, furans, weak acids (Table 4.2) and lower molecular weight phenolic compounds (Table 4.3). The main sugar in the liquor fraction was xylose (9.1 g/L), together with a high concentration of acetic acid (6.7 g/L) and furfural (2.3 g/L).

Table 4.1 Sugarcane bagasse pressed solids (filter cake)

Sugars (%)		Lignin (%)		Moisture (%)
Cellobiose	**Glucose**	**Acid soluble**	**Acid insoluble**	
5.5 ± 0.5	48.4 ± 1.5	3.5 ± 0.19	30.7 ± 1.3	6.2 ± 0.18

Table 4.2 Sugarcane bagasse liquor (sugar degradation by-products) (g/L)

Cellobiose	Glucose	Xylose	Arabinose	Acetic acid	Formic acid	HMF[1]	Furfural
0.47 ± 0.008	0.91 ± 0.4	9.1 ± 0.04	0.59 ± 0.007	6.7 ± 0.04	1.1 ± 0.2	0.27 ± 0.005	2.3 ± 0.02

[1] HMF: Hydroxymethyl furfural

Table 4.3 Sugarcane bagasse liquor (Phenolic compounds) (mg/L)

3,5 DHBA[2]	Vanillic acid	Syringic acid	Vanillin	SA[3]	Ferulic acid	CON[4]
22.2 ± 0.06	21.4 ± 0.16	46.8 ± 0.14	102 ± 0.08	4.5 ± 0.06	3.1 ± 0.004	5.6 ± 0.03

[2] 3,5 DHBA: 3,5 Dihydroxybenzoic acid, [3]SA: Syringaldehyde, [4] CON: Coniferyl aldehyde

4.4 DISCUSSION

Detoxification could potentially alleviate the inhibition/deactivation induced by phenolic compounds during the enzymatic hydrolysis of cellulosic biomass. Reducing agents, in particular sulfur oxyanions, have been shown to be efficient *in situ* detoxifying agents. This implies that detoxification can be performed in the same vessel as enzymatic hydrolysis and fermentation, thus eliminating the need for separate processes and reducing operating costs (Alriksson et al. 2011). Sulfur oxyanions were proposed to act by sulfonating the inhibitory compounds and mainly reacting with aldehyde functional groups on the surface of the inhibitor at lower temperature or reducing the inhibitor to its corresponding alcohol under reducing conditions (Cavka et al. 2011, Soudham et al. 2011). This neutralises or renders the inhibitor unreactive towards the hydrolytic enzymes.

In a previous study, we showed that tannic acid was the strongest inhibitor among seven phenolic compounds tested for their impact on cellulolytic hydrolysis (Mhlongo et al. 2015). The other phenolic compounds that showed a significant deactivation profile contained an aldehyde functional group, such as coniferyl aldehyde and syringaldehyde. The current study focused on the effect of detoxification on compounds containing an aldehyde functional group, such as coniferyl aldehyde and syringaldehyde; tannic acid was excluded due to its complex structure and lack of an aldehyde group. The inhibiting model compounds

(coniferyl aldehyde and syringaldehyde) were observed to react with the detoxifying agents (sodium sulfite or sodium dithionite). The sulfur oxyanions are suggested to affect the double bond in coniferyl aldehyde and change the hydrophobic surface of the inhibitor to a hydrophilic surface. Under reducing conditions, the aldehyde functional groups of the inhibitors are reduced to their corresponding alcohols, whereas the inhibitors are sulfonated at lower temperatures (Cavka et al. 2011).

The relatively high cellulose loading (10% Avicel) might have masked the effect of the phenolic inhibitors (evaluated at 1 mM concentrations), as the recombinant cellulases also did not perform well at the high substrate loading. Mhlongo et al. (2015) reported 7.6 g/L glucose released from 1% Avicel in 48 hr, whereas only 6.5 g/L of glucose was released from 10% Avicel in this study, suggesting reduced catalysis in a high solids loading.

The effect of inhibition and/or detoxification was further investigated by quantifying the residual activity of TeCel7A-TrCBM (Bu et al. 2011, Kont et al. 2013, Teugjas and Väljamäe 2013). Coniferyl aldehyde inhibited both glucose production and TeCel7A-TrCBM activity (Fig. 4.1). Although there was a substantial improvement in glucose production in the presence of sodium dithionite, the TeCel7A-TrCBM residual activity was similar to reactions containing only coniferyl aldehyde, only detoxifying agent or both. Syringaldehyde displayed a more profound inhibition of TeCel7A-TrCBM residual activity, which was significantly improved with the addition of sodium sulfite or dithionite (Fig. 4.2). These results support our earlier findings that 1 mM syringaldehyde did not directly impact Avicel hydrolysis by a complex mixture of cellulases, but it reduced the enzyme activity of TeCel7A-TrCBM three-fold (Mhlongo et al. 2015). In addition to unproductively binding of cellulases to the inhibitors during hydrolysis, the cellulases could also be bound to the substrate. It is possible that the decrease observed in the TeCel7A residual activity was sequestered due to adhesion to the solid fraction as well as binding to the inhibitor.

The results suggested that the detoxification effect of sulfonates was more profound towards soluble inhibitors (either in the liquid hemicellulose fraction or individual inhibitor compounds) than with insoluble inhibitors (in the filter cake fraction). A biological detoxification strategy using laccase showed that laccase reacted with the inhibitor compounds, but no clear improvement in glucose production was observed (Fig. 4.3).

All of the above-mentioned experiments were conducted on Avicel, which is a pure cellulose substrate. The question remained as to whether similar effects will be displayed in the presence of a natural substrate, such as those represented by bagasse filter cake and the pretreatment liquor fraction. An enzymatic hydrolysis experiment with detoxifying agents was conducted using bagasse filter cake and/or Avicel spiked with pretreatment liquor (containing soluble inhibitors). The results showed that detoxification with sulfonates did not assist in the hydrolysis of bagasse filter cake (Fig. 4.4). This could be ascribed to a number of factors such as feedback inhibition from sugars in the bagasse filter cake or inhibition by lignin. The complexity of the bagasse filter cake may add to the inhibition problem that may require more than just a detoxification strategy. Since only recombinant cellulases were used in this study, the inclusion of accessory enzymes for detoxification of filter cake could be investigated in future studies.

Substantial improvement in the hydrolysis was observed when the detoxified pure cellulose was spiked with bagasse pretreatment liquor, in particular for treatment with sodium dithionite. The application of sodium dithionite and sodium sulfite has been shown to improve the fermentability of bagasse hydrolysate (Soudham et al. 2011). These sulfur oxyanions react predominantly with the aldehyde functional groups in the inhibitor mixture (Cavka et al. 2011). The sulfur oxyanions may also sulfonate the inhibitors, thus converting it to highly hydrophilic, charged molecules (Cavka and Jönsson 2013). Lignosulfonate also had a positive effect when added to the liquid hydrolysate, but not for pure Avicel or bagasse filter cake. Lignosulfonate functions as a surfactant and its properties have been exploited commercially in the surface modification of products such as dispersants and plasticizers (Wang et al. 2013). There are two competing processes that involve lignosulfonates; on pure cellulose substrates: they adsorb cellulases and thus reduce their availability and activity, or they serve as surfactants on solid substrates where they block lignin from adsorbing cellulases. We observed a similar pattern with the addition of sulfur oxyanions to pure Avicel in the absence of inhibitors: detoxifying agents resulted in a substantial inhibition of cellulase activity suggesting that these agents act as inhibitors of cellulose hydrolysis in the absence of an inhibitor compound.

In situ detoxification using sulfur oxyanions can be performed without adjusting the pH or changing the reaction temperature and does not result in sugar degradation. These are beneficial properties in process configurations such as simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP) (Soudham et al. 2014). This has

a potential to open new avenues for performing lignocellulosic conversion processes in one fermentation vessel in the presence of inhibiting compounds and thus reducing the cost of enzyme hydrolysis of cellulosic biomass.

4.5 CONCLUSION

Reducing agents such as sodium dithionite or sodium sulfite were previously reported as potential chemical compounds for *in situ* detoxification of pretreated biomass prior to enzyme hydrolysis (Alriksson et al. 2011). This study showed that these compounds reacted with single inhibitor compounds containing an aldehyde functional group. The detoxification effect was not obvious in the presence of coniferyl aldehyde, but glucose production from Avicel correlated with higher residual activity of TeCel7A-TrCBM in the presence of syringaldehyde. The positive impact of sulfur oxyanions was also observed when sugarcane bagasse was used, more particularly in detoxifying soluble inhibitors in the hemicellulose fraction, with sodium dithionite that showed an exceptional improvement in substrate hydrolysis under these conditions. In addition to the positive effects on hydrolysis observed with sodium dithionite and sodium sulfite, lignosulfonate also showed positive effect in enhancing the hydrolysis reaction in the presence of pretreatment liquor. Application of lignosulfonate as a detoxifying agent during enzymatic hydrolysis has significant scientific and practical implications and could allow for simplification of the biorefinery process.

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Chapter 5

RESEARCH RESULTS III

THE ROLE OF CARBOHYDRATE BINDING MODULES IN NON-PRODUCTIVE BINDING OF CELLOBIOHYDROLASES

The role of carbohydrate binding modules in non-productive binding of cellobiohydrolases

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ABSTRACT

Bimodular cellulases contain hydrophobic residues exposed on the surface of the catalytic domain and the carbohydrate binding module (CBM). The presence of a CBM enhances the binding of cellulases to cellulose fibres, thereby facilitating efficient hydrolysis. Inevitably, these hydrophobic residues may also contribute to non-productive binding of cellulases to inhibitory compounds released during the pretreatment of cellulosic biomass, which may result in the inhibition/deactivation of the cellulases. In this study, a *Talaromyces emersonii* Cel7A (with or without a C-terminally fused *Trichoderma reesei* CBM) was exposed to selected inhibitor compounds and subjected to differential scanning fluorimetry. The protein melt curves showed that acetic acid immediately unfolded the protein and exposed the buried hydrophobic regions, without binding to the hydrophobic regions in the unfolded protein. In contrast, furan aldehydes and phenolic compounds were bound to the exposed hydrophobic regions of the protein in the pre-transitional and unfolding state. We suggest that hydrophobic interactions mainly facilitate the binding of furan and phenolic compounds to the enzymes whereas electrostatic interactions resulted in the unfolding of the enzymes by acetic acid. The study concluded that the presence of the CBM did not have a substantial impact on substrate binding or thermal stability of the cellulases since the enzymes maintained their protein conformation. However, inhibitors such as furans and phenolic compounds could shield the hydrophobic residues required for binding to cellulose substrates and thus negatively impact substrate binding, therefore reducing its hydrolytic efficiency.

Keywords: cellulases, inhibitors, hydrophobic interactions, substrate binding, differential scanning fluorimeter, carbohydrate binding module (CBM)

5.1 INTRODUCTION

Cellobiohydrolases are the most important single enzyme components involved in the enzymatic hydrolysis of cellulose (Jeoh et al. 2008). *Trichoderma reesei* Cel7A is the most studied cellobiohydrolase, which represents approximately 60% of the total protein secreted by *T. reesei*. The TrCel7A protein is typically a bimodular domain protein consisting of the catalytic domain connected to a carbohydrate binding module (CBM) through a highly-glycosylated linker (Palonen et al. 2004, den Haan et al. 2013, Le Costaouec et al. 2013). Carbohydrate binding modules are protein domains found in hydrolytic and non-hydrolytic proteins involved in cellulose degradation and are defined as contiguous amino acid sequences with discrete folds and carbohydrate binding activity (http://www.cazy.org/). These domains have been broadly defined based on their diverse ligand specificity during sugar binding events (Oliveira et al. 2015). CBMs are categorised into 66 families and only members of families 1, 6 and 63 have cellulose binding affinity. The most studied is the *T. reesei* CBM (TrCBM), which belongs to family 1 and consists of a planar face with conserved amino acid moieties that target crystalline cellulose fibrils (Varnai et al. 2014). They have a β-sandwich fold characterized by two β-sheets consisting of three to six antiparallel β-strands (Oliveira et al. 2015). The family 1 TrCBMs are composed of 30 to 40 highly conserved amino acid residues (e.g. tyrosine residues Y5, Y31, Y32) and charged residues (e.g. Q7, Q34 and N29) that are critical for binding to surfaces. Three aromatic amino acids in the CBM are arranged to align with alternating β(1-4)-linked glucose units on the cellulose surface (Varnai et al. 2014, Strobel et al. 2015).

The CBM plays a significant role in directing the enzyme to bind to crystalline cellulose; this increases the concentration of the catalytic domain on the surface of cellulose fibrils and thus the catalytic efficiency of the enzyme (Gao et al. 2014). While the primary purpose of the aromatic residues in the CBM surface is to facilitate productive binding to cellulose, they are also involved in non-productive adsorption of cellulases onto lignin residues through hydrophobic interactions. The role of CBMs in non-productive adsorption is dependent on the pH and the functional groups on the lignin surface (Rahikainen et al. 2013, Strobel et al. 2015). Lignin is a hydrophobic aromatic polymer in lignocellulosic biomass, primarily composed of three precursors, namely *ρ*-coumaryl, coniferyl and sinapyl alcohol (Qin et al. 2014, Sammond et al. 2014). Since lignin limits the access of cellulases to sugar polymers in lignocellulosic substrates, enzymatic hydrolysis requires a pretreatment process that delocalises lignin and thus improve access to sugar polymers. In addition, pretreatment

partially solubilises lignin to various low-molecular weight lignin monomers that also bind cellulases non-productively (Kim et al. 2015).

Non-productive adsorption of cellulases onto lignin residues triggers changes in the protein conformational structure and intensifies the protein-surface interaction, which could lead to denaturation (Gao et al. 2014). Generally, proteins have a hydrophobic core and a hydrophilic surface that interact with the surface environment. However, most cellulases are relatively rich in hydrophobic amino acids that are randomly or uniformly distributed on the protein surface. In addition, the surface of cellulases is less polar than water and rich in functional groups such as hydroxyl and carboxyl groups, which facilitate the formation of multiple bonds including hydrogen bonds, charged or partially charged interactions. All these surface characteristics of cellulases are suggested to contribute to non-productive binding to other surfaces, such as lignin or lignin residues (Qin et al. 2014). Non-productive binding is largely facilitated by hydrophobic interactions between the charged or partially charged aromatic acid residues on both lignin and enzyme surfaces. The interaction may result in irreversible adsorption, denaturation and ultimate loss in enzyme available for hydrolysis (Varnai et al. 2014, Rahikainen et al. 2013). As a consequence, a higher enzyme load is required to compensate for the enzyme lost through non-productive binding, which increases the overall cost of enzymatic hydrolysis (Strobel et al. 2015).

The adsorption of cellulases onto a cellulose surface impacts the hydrolysis rate (Le Costaouec et al. 2013). In an ideal situation, cellulases should exhibit a quick initial adsorption that is followed by a slow adsorption-desorption of the enzyme components to promote processivity. However, enzymes could also be non-productively bound on the surface of lignin, or they may be inhibited and/or deactivated, which slows down enzymatic hydrolysis. However, differentiating between productive and non-productive binding of cellulases in complex substrates has not been possible (Varnai et al. 2011).

Rahikainen et al. (2013) reported that the full-length TrCel7A binds more tightly to lignin compared to its catalytic core domain lacking the linker and CBM. The full length TrCel7A has a fast initial adsorption phase followed by a slower desorption phase, while the catalytic domain lacking a linker and CBM has a slower adsorption phase and a faster desorption phase (Varnai et al. 2011, Varnai et al. 2013). This suggests that CBM together with the

linker region are required for the initial fast adsorption phase (Palonen et al. 2004, Rahikainen et al. 2013). The adsorption patterns of cellulases to either cellulose or lignin surfaces also differ depending on the plant source where that particular cellulose is derived from (Rahikainen et al. 2011). In earlier studies, it was generally accepted that the catalytic core had reduced binding affinity for cellulose compared to the full length protein containing the CBM. However, recent findings suggest that comparable hydrolysis yields could be obtained with either intact Cel7A or its catalytic core domain lacking CBM at high substrate concentrations (Varnai et al. 2013, Le Costaouec et al. 2013).

Experimental evidence thus suggests that the CBM may be more important in dilute systems for targeted binding to cellulose surfaces than in high substrate concentrations (Varnai et al. 2013). The lack of a CBM in cellulases can therefore be counterbalanced by reducing the amount of water in the hydrolytic system to increase the relative concentration of cellulase on the cellulose surface. Some reports concluded that the presence of the CBM decreases the specific activity of cellulase towards cellulose hydrolysis (Pakarinen et al. 2014). However, due to the prevalence of cellulases naturally possessing CBMs, studies in dilute systems have favoured these cellulases as they were believed to be more efficient than their counterparts lacking a CBM. There are less systematic studies on the properties of naturally occurring CBM-less cellulases (Le Costaouec et al. 2013). Furthermore, the structural complexity of cellulases makes it difficult to understand the mechanism of the CBM in the binding process (Guo et al. 2014). The aim of this study was compare the extent to which a *Talaromyces emersonii* Cel7A (TeCel7A) lacking the CBM and a TeCel7A fused to a TrCBM binds to inhibitory compounds and the effect of this binding on the stability of these proteins. A direct binding approach using differential scanning fluorimeter (DSF) was used to monitor the temperature-induced transitions in TeCel7A and its fusion protein derivative (TeCel7A-TrCBM).

5.2 MATERIALS AND METHODS

5.2.1 Yeast strains, media and cultivation conditions

All chemicals, media components and supplements used in this study were of analytical grade and were sourced from Sigma-Aldrich (South Africa) unless stated otherwise. The *Saccharomyces cerevisiae* Y294 yeast strain expressing TeCel7A with a carbohydrate binding domain (CBM) fused to its carboxyl terminal, Sc[Tecbh1-TrCBM-C] previously

constructed by Ilmen et al. (2011) was maintained at 30ºC on YPD agar plates (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) and was used for the production of extracellular enzymes. To obtain a high concentration of extracellular protein, Sc[Techb1-TrCBM-C] was grown in 8 x 2 L Erlenmeyer flasks containing 500 mL double-strength SC media (3.4 g/L yeast nitrogen base w/o amino acids and ammonium sulphate, 10 g/L ammonium sulphate, 20 g/L glucose and supplemented with amino acids as required), buffered at pH 6.0 with 0.17 M succinate buffer.

5.2.2 Partial purification of TeCel7A-TrCBM

Partial purification of TrCel7A-TrCBM was carried out as reported by Den Haan et al. (2013). After 4 days of cultivation in SC medium at 30°C with orbital shaking at 200 rpm, cultures were centrifuged and the supernatant filtered through 0.45 µm glass fibre filters (Millipore, Molsheim, France). The filtrate was concentrated to 800 mL with the Minitan system (Millipore, Molsheim, France) using a stacked 5 kDa ultrafiltration membrane, followed by the Amicon ultrafiltration system (Millipore) fitted with a 30 kDa cut-off membrane to concentrate it to 100 mL. Thereafter, the sample was washed with two volumes of 50 mM Na-acetate buffer (pH 5.0) and concentrated to a final volume of 50 mL with the Amicon system, followed by fractionation with an increasing gradient (50 to 100%) of ammonium sulphate at 4ºC. All fractions were collected and dialysed with 20 mL of 50 mM sodium acetate buffer (pH 5.0) in the Amicon system with a 30 kDa cut-off membrane to remove ammonium sulphate. The proteins in the respective fractions were analysed by sodium dodecyl sulphate-acrylamide gel electrophoresis (SDS-PAGE) using a 10% (w/v) acrylamide gel and quantified using a BCA (bisinchoninic acid) protein assay kit (Pierce Chemical Company,, Rockford, Illinois, USA) with bovine serum albumin (BSA) as a standard.

5.2.3 Partial papain proteolysis

A partially purified TeCel7A-TrCBM protein was treated with papain enzyme (*Papaya latex*, Merck, South Africa) at a protein-papain ratio of 200:1 (w/w) for 2 hours at 30ºC while shaking at 200 rpm. Papain was pre-activated in 50 mM phosphate buffer (pH 6.3) with 2 mM EDTA and 5 mM cysteine-HCl for 30 minutes (Lemos et al. 2000, Hall et al. 2011, Lima et al. 2013). After partial papain proteolysis, equal volume of 50 mM sodium acetate buffer (pH 5.0) was added to the sample and filtered with a 30 kDa cut-off membrane to remove the CBM fraction. Both the partially purified sample and filtrate fractions were analysed for

enzyme activity (using *p*-nitrophenyl-β-D-cellobiosidase as substrate) and protein content (using the Pierce protein assay) as per the suppliers' instructions. SDS-PAGE gels (10% and 20%) were used to confirm the proteolytic cleavage of the CBM.

5.2.4 Hydrolysate inhibitor compounds

Selected model compounds representing by-products from lignocellulosic biomass were added at concentrations as described in the previous chapter. The selected compounds included acetic acid, 5-furfural; syringaldehyde, vanillin and 4-hydroxy-3-methoxy-cinnamaldehyde (coniferyl aldehyde). The model compounds were evaluated at 25%, 50%, 75% or 100% concentrations as previously described in Chapter 3. The inhibitor compounds were prepared fresh and filtered with a 0.2 µm Acrodisc® 25 mm PF syringe filter (Pall Corporation, USA) before use.

5.2.5 Differential scanning fluorimeter (DSF)

The transition states of protein unfolding were measured using the DSF technique. Aliquots of 20 µL protein sample were prepared by mixing 2.5 µL of 8-fold concentrated SYPRO orange dye (Sigma-Aldrich, St Louis, Missouni, USA) with 12.5 µL of protein (to a final concentration of 47 µg/mL) in 50 mM sodium acetate buffer, pH 5.0. For protein-ligand binding samples, a 5-fold concentrated model inhibitor solution was added at 25% to 100% of the final concentration. The fluorescence was measured using a temperature gradient from 25°C to 99°C with 0.5°C increments in an Applied Biosystems 7500 Fast RT-PCR system (Applied Biosystems, California, USA) with dye detection (λ_{em}=477 nm/λ_{em}=549 nm). The change in fluorescence intensity expressed as arbitrary units (A.U.) was obtained by determining the difference between the highest and the lowest fluorescence intensity in the DSF curve (Niesen et al. 2007) using Graph Pad Prism (GraphPad Software, San Diego, California) and Microsoft Excel (2010).

5.3 Results

5.3.1 Partial papain proteolysis

Limited papain proteolysis of TeCel7A-TrCBM (~65 kDa) yielded a ~56 kDa catalytic core fragment as observed in a 10% SDS-PAGE gel (Fig. 5.1), with no detectable cleaved linker or CBM fragment (~9 to 10 kDa), not even on a 20% SDS-PAGE gel (data not shown).

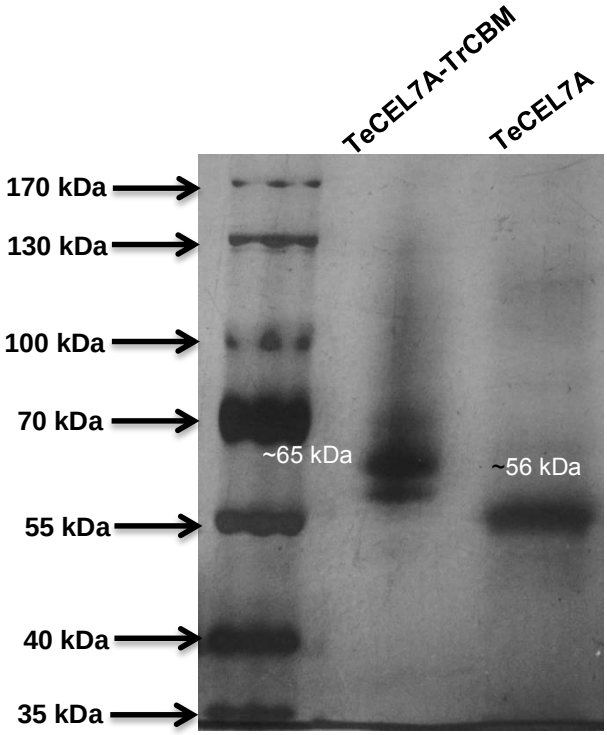

Fig. 5.1 Reducing 10% SDS-PAGE gel of the full-length protein (TeCel7A-TrCBM) or with the TrCBM and linker removed by papain proteolysis (TeCel7A). The gel was visualised with silver staining and the molecular weight size markers are indicated on the left.

5.3.2 Differential scanning fluorimeter (DSF)

With DSF, an ideal melt curve exhibits a sigmoidal curve with three distinct transitional states: the first state is a flat pre-transition baseline where the native protein is in a well-folded state, followed by a steep transition that signals rapid unfolding of a protein. After the maximum fluorescence intensity is reached, re-aggregation of the hydrophobic regions and thus quenching of the dye results in a slow decrease in signal (Seabrook and Newman 2013). In the present study, both the full-length protein (TeCel7A-TrCBM) and its catalytic domain (TeCel7A) had a short pre-transition state in the presence of acetic acid (Fig. 5.2A).

The catalytic domain displayed a higher fluorescence intensity in the pre-transitional state compared to the full-length TeCel7A-TrCBM. A steep unfolding state was observed for TeCel7A-TrCBM, while the unfolding state was less steep for the catalytic domain. There was no substantial difference in the fluorescence intensity for the full length protein and its catalytic domain only. However, acetic acid had a greater impact on the thermal stability of the protein as the melting temperature of both TeCel7A-TrCBM and TeCel7A shifted to approximately 49°C and had a higher fluorescence compared to the control protein without inhibitors, which displayed a melting temperature of approximately 65°C.

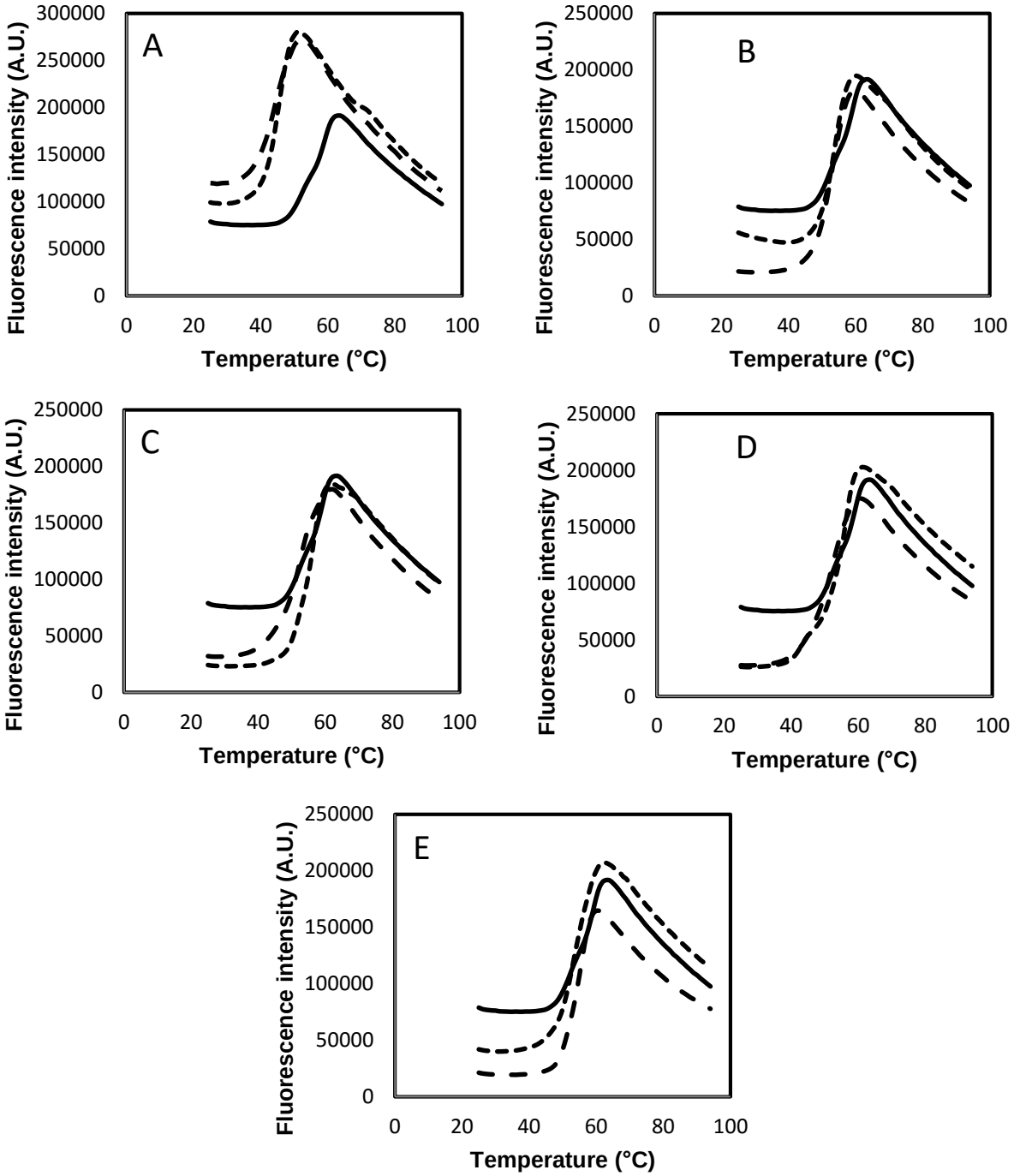

Fig. 5.2 Thermal transition of TeCel7A-TrCBM (square dotted line) and TeCel7A (dash line) in the presence of (A) 75 mM acetic acid, (B) 30 mM furfural, (C) 1 mM syringaldehyde, (D) 1 mM coniferyl aldehyde and (E) 1 mM vanillin. The control protein is a full-length TeCel7A-TrCBM in a reaction without inhibitors (solid line). The results represent an average of three experimental repeats.

In the presence of furfural, the TeCel7A catalytic domain showed a flat pre-transition state with reduced fluorescence intensity compared to the full-length and control proteins (Fig.

5.2B). However, the unfolding states (melt curves) for TeCel7A-TrCBM and TeCel7A were similar, except that the thermal stability for TeCel7A was decreased. The effect of furfural on the thermal stability of TeCel7A-TrCBM and TeCel7A was negligible when compared to the control protein.

A typical sigmoidal curve transition state was observed with TeCel7A-TrCBM in the presence of syringaldehyde. The TeCel7A protein had a shorter pre-transitional state and a steep unfolding state (Fig. 5.2C). For both the TeCel7A-TrCBM and the TeCel7A proteins, the fluorescence intensity was lower than for the control protein. Syringaldehyde did not have a substantial effect on the stability of the inhibited proteins, but the melt curve of the full-length protein was similar to TeCel7A lacking the CBM. In the presence of coniferyl aldehyde, the full length protein (TeCel7A-TrCBM)) and its catalytic core (TeCel7A) had an interrupted sigmoidal curve (Fig. 5.2D). A substantial decrease in the thermal stability of the TeCel7A protein lacking the CBM was observed. Both TeCel7A-TrCBM and TeCel7A had a typical sigmoidal curve in the presence of vanillin (Fig. 5.2E), with a substantially reduced fluorescence intensity of the catalytic domain.

5.4 Discussion

This study investigated the mechanism(s) involved in the inhibition and/or deactivation of cellulases by inhibitor compounds released during the pretreatment of lignocellulosic biomass. We also investigated the role of the Cel7A catalytic and binding domains in inhibition/deactivation in the presence of soluble inhibitors previously reported (Mhlongo et al. 2015). Other inhibitor compounds such as tannic acid were excluded in this study since they interfere with the hydrophobic dye. The Cel7A protein was selected as a representative cellulase as it is instrumental in the hydrolysis of highly crystalline cellulose and is the most studied cellulase for biofuel applications. The *T. reesei* Cel7A consists of three domains, namely a 434-amino acid catalytic domain, a 36-amino acid carbohydrate binding module (CBM) and a heavily glycosylated 24-amino acid linker that spatially separates the catalytic domain and the CBM (Hall et al. 2011). The CBM and linker domain can be removed by either enzymatic proteolysis or via a recombinant DNA strategy. In this study, a proteolytic approach (partial papain treatment) was used to remove the TrCBM moiety fused to the TeCel7A. The recombinant strategy is usually preferred when a pure CBM-containing fraction is required for further applications as it avoids contamination of CBM with residual

catalytic activity (Oliveira et al. 2015). However, for the purpose of this study, the CBM fraction was not of interest and the proteolytic cleavage was the best option.

The proteolytic cleavage of TeCel7A-TrCBM (~65 kDa) yielded a catalytic domain of approximately 56 kDa. This is in agreement with the MALDI-MS results reported for the proteolytic cleavage of TrCel7A where the intact protein had a molecular weight of 61.8 kDa and the catalytic domain was between 52.4 and 53.1 kDa (Jeoh et al. 2008). Similar product concentrations were obtained in this study for protein:papain ratios of 50:1, 100:1 or 200:1 at different incubation times. The proteolytic cleavage of Cel7A occurs between two glycine residues (Gly439 and Gly440) at the C-terminal end of the catalytic domain and separates the catalytic domain from the linker peptide and the CBM (Jeoh et al. 2008). It was reported in the late 1980s that the partial papain proteolysis action on cellobiohydrolases indicates the presence of hinge regions linking the core fragment to the terminal glycopeptides (linker and CBM) (Tomme et al. 1988). The protein band for the intact TeCel7A-TrCBM appeared as a broader and more diffused band compared to the catalytic domain (Fig. 5.1). This is typical of proteins with polydispersity of molecular weight due to glycosylation (Jeoh et al. 2008), which could be ascribed to the highly *o*-glycosylated linker domain.

Removal of CBMs has been reported to substantially decrease enzyme activity on insoluble substrates, but no effect on soluble substrates has been reported (Rahikainen et al. 2013). Ilmen et al. (2011) found that attachment of a TrCBM to a well-secreted cellobiohydrolase (TeCel7A) that naturally occurs as a CBM-less protein, resulted in a fusion protein (TeCel7A-TrCBM) that performed better in Avicel hydrolysis than a non-fused protein (TeCel7A). However, a review of genomic data indicated that a number of cellulases lack the binding domain (CBM) (Varnai et al. 2014). There is also evidence that the catalytic domains lacking CBMs proceed along cellulose chains with a similar speed than the intact enzyme with comparable turnover numbers towards cellulose (Palonen et al. 2004, Pakarinen et al. 2014). In practical industrial applications where there is high substrate loading and lower amount of water in the hydrolysis reactions, the catalytic efficiencies of enzymes with or without the CBM were comparable (Varnai et al. 2014). This suggests that the critical function of the CBM is to increase the concentration of cellulases on the cellulose surface, which may not be necessary in enzymatic hydrolysis reactions with a high substrate loading.

Non-productive adsorption is mainly facilitated by hydrophobic bonding between the hydrophobic regions on the cellulases and the surface of the cellulose fibres (Palonen et al. 2004, Rahikainen et al. 2013). In this study, we used a differential scanning fluorimeter where a fluorescence dye binds to exposed hydrophobic regions of the protein and the fluorescence intensity on the protein melt curve indicates the level of protein unfolding. The melt curves (Fig. 5.2) suggest that acetic acid destabilised the protein structure even at lower temperatures. The inhibited proteins had a shorter pre-transition state compared to the control protein, suggesting that acetic acid immediately disrupted the protein and exposed hydrophobic regions. Typically, proteins in the pre-transitional state are well-folded and have a flat pre-transitional state as was observed with the control protein. We speculate that acetic acid does not bind to the exposed hydrophobic regions of the proteins during the unfolding state, thus allowing the fluorescence dye to bind to both the inhibited proteins as opposed to the control protein. This suggests that the inhibition/deactivation effect of acetic acid observed in our previous study, was not dependent on the hydrophobic interaction, but rather the ionic strength of the reaction.

Binding of furfural during the pre-transitional state did not impact the native folding of the protein, since both the inhibited proteins had a flat pre-transition state (Fig. 5.2). The fluorescence intensity for TeCel7A-TrCBM during the pre-transitional state was higher than for TeCel7A, but lower than for the control. Since the CBM contains three conserved hydrophobic residues on its surface (Rahikainen et al. 2013), the CBM could increase the number of hydrophobic regions on the surface of the protein. Some of the hydrophobic regions in the full length protein may be bound by furfural, which decreased fluorescence relative to the control protein (without furfural inhibitor).

Binding of furfural to either TeCel7A-TrCBM or TeCel7A had a minor effect on the thermal stability of these proteins (Fig. 5.2). This suggests that furfural may bind to the Cel7A protein and shield the exposed hydrophobic regions without affecting protein stability. This is in agreement with our earlier findings that furfural does not result in a substantial loss of Cel7A activity (Mhlongo et al. 2015), although shielding of hydrophobic regions may have a negative impact on the binding of Cel7A and therefore its hydrolytic efficacy. A similar effect on the melt curve was observed with TeCel7A-TrCBM and TeCel7A when inhibited by phenolic aldehydes, i.e. syringaldehyde, coniferyl aldehyde and vanillin. Removal of the CBM had a more notable effect in the presence of coniferyl aldehyde or vanillin, with substantially reduced fluorescence intensities and displayed lower melting points for

TeCel7A than TeCel7A-TrCBM. It was reported that TeCel7A function is fully dependent on its catalytic domain and that the enzyme can perform its functions without the CBM (Brady et al. 2015). Our results suggest that the addition of the CBM increases the hydrophobic binding affinity of the protein to the substrate.

5.5 Conclusion

Previous studies concluded that cellulases with CBMs bind more efficiently to cellulose substrates than CBM-less cellulases (Varnai et al. 2011). However, other reports suggest that the presence of a CBM does not confer increased activity on the cellulase, but rather increases the proximal concentration of cellulases on the surface of cellulose in dilute systems, with an insignificant effect in systems with a high solid content. In this study, we observed that the presence or absence of the CBM did not have a substantial impact on the binding and/or thermal stability of the inhibited protein. Furthermore, hydrophobic interactions facilitated binding of the enzyme to furan aldehydes and phenolic compounds, while the impact of acetic acid was likely to occur via electrostatic interactions. It was thus concluded that the binding of cellulases to inhibitor compounds via hydrophobic interactions did not disrupt the protein conformation, but the hydrophobic residues required for binding of the enzyme to the cellulose substrate could have been shielded by the binding of the inhibitor compound to the hydrophobic cellulose residues.

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Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS

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6.1 GENERAL DISCUSSION

In recent years, there has been a growing emphasis on the development of sustainable green technologies for the production of alternative energy sources. Ethanol is a liquid fuel that can be sustainably produced from lignocellulosic biomass for second-generation bioenergy (van Zyl et al. 2011, Mohanram et al. 2013). Lignocellulosic biomass is abundant, cheap and sustainable compared to first-generation bioenergy that relies on feedstocks that also serve as food sources, such as corn (Sims et al. 2010). Despite the significant progress made thus far, the full-scale commercialisation of biofuels based on lignocellulosic biomass still faces major techno-economic challenges (Cardona et al. 2010). One of the most challenging steps is the enzymatic hydrolysis of cellulosic biomass (Vermaas et al. 2015). Effective hydrolysis of cellulosic biomass requires a number of cellulases, which are commonly sourced from wood-rotting fungi such as *Trichoderma, Penicillium* and *Aspergillus*, which bind and synergistically hydrolyse lignocellulosic substrates (Sims et al. 2010). The enzymes that synergistically hydrolyse cellulose are grouped as exoglucanases or cellobiohydrolases, endoglucanases and β-glucosidases (Mello and Polikarpov 2014).

The combined action of cellulases on lignocellulosic biomass releases glucose, which can be subsequently fermented to ethanol (van Zyl et al. 2011). However, the rate of cellulase binding to cellulose is limited by the recalcitrance of the lignocellulosic material (Zheng et al. 2013). The recalcitrance problem is typically overcome by applying a pretreatment process that opens up the lignocellulose structure for improved access of the hydrolytic enzymes. Various pretreatment options have been proposed to improve the accessibility of cellulases, but inhibitory by-products and insoluble lignin remain in pretreatment slurries, regardless of the pretreatment process. By-products that are typically released during biomass pretreatment are weak acids, furans and phenolic compounds. Part of the non-degraded (insoluble) lignin remains in contact with carbohydrate polymers (Qin et al. 2016) and thus hamper the subsequent enzymatic hydrolysis step and reduce the sugar yield. In an ideal situation, a desired pretreatment is one that yields pure cellulose that would allow for optimal enzyme activity. However, obtaining a pure cellulosic substrate will not be economical for industrial production of biofuels since it requires a substantial amount of energy and is often associated with a significant loss in fermentable sugars.

It is this clear that the enzymatic conversion of biomass will have to proceed in the presence of a certain amount of pretreatment inhibitory products, which necessitates a proper understanding of the impact of these inhibitory by-products on enzymatic hydrolysis (Zheng et al. 2013). A number of studies suggest that phenolic compounds are stronger inhibitors of cellulases than furans or weak acids. Phenolic compounds can result in the precipitation and irreversible inhibition of cellulases in the hydrolytic system. However, there is still limited knowledge about inhibition mechanisms and more studies are required for a better understanding of the mechanism of lignin and/or phenolic inhibition of cellulases in order to design efficient biomass conversion processes (Qin et al. 2016).

The broader aim of the study was to investigate the mechanism(s) of interaction between recombinant cellulases (TeCel7A-TrCBM, TrCel5A and SfCel3A) and by-products (phenolic compounds, weak acids and furan aldehydes) typically released during the pretreatment of lignocellulosic biomass. Pretreatment by-products are widely known to inhibit/deactivate enzymatic hydrolysis and fermentation steps during biomass conversion to biofuels. A systematic approach was thus followed to investigate the role of individual lignocellulose-associated inhibitor compounds in the inhibition and/or deactivation of cellulases (TeCel7A-TrCBM, TrCel5A and SfCel3A) individually or in combination (Chapter 3). We further evaluated detoxification strategies that could be used to alleviate the inhibition/deactivation effect (Chapter 4) and studied the possible mechanism(s) facilitating the inhibition and/or deactivation (Chapter 5).

Weak acids were observed to be strong inhibitors of cellulases that had an immediate effect in the hydrolysis reactions. SfCel3A was strongly inhibited by compounds such as formic, tannic and acetic acids, but no substantial effect on SfCel3A was observed with furans and phenolic compounds (Chapter 3). Using TeCel7A as a representative cellulase, we showed that acetic acid unfolded the cellulases, but did not bind to the exposed hydrophobic regions in the protein (Chapter 5). The pH of the hydrolysis reaction decreased as the concentration of acetic or formic acid increased, but this was not the case with tannic acid and cinnamic acid. We hypothesize that the mechanism of inhibition by acetic acid and probably formic acid could be through electrostatic interactions rather than hydrophobic binding.

Beta-glucosidases are known to form a dimer in solution and have pairs of distal hydrophobic patch regions (Sammond et al. 2014). These hydrophobic patches are far from the β-glucosidase active site, which leaves the active site available to interact with a cellulosic substrate. We speculated that the phenolic compounds and furans bind to the hydrophobic areas on the β-glucosidase, but without a significantly effect on its activity due to the distance between the patches and the active site. Similar observations were reported with the *Aspergillus niger* β-glucosidase (AnCel3A), which was irreversibly adsorbed to lignin whilst maintaining its activity (Sammond et al. 2014). The authors suggested that binding of AnCel3A to phenolic compounds could assist in anchoring AnCel3A to the substrate in the absence of a CBM moiety. In view of these reports, we speculated that tannic acid binds to the SfCel3A through hydrophobic interactions. However, the bulky size of this molecule could have resulted in steric hindrance as a strong inhibition effect was observed in the reaction catalysed by SfCel3A in the presence of tannic acid. We were unable to confirm this proposed mechanism with tannic acid since this large molecule interfered with the fluorescence dye in the DSF technique used to study the mechanism of binding (Chapter 5). An alternative method, the internal reflection fluorescence spectroscopy (TIRF), can be used to study the interaction between cellulases and tannic acid or other hydrophobic compounds in hydrolysate to clearly elucidate the mechanism of binding.

The polymeric phenols (represented by tannic acid) strongly inhibited all cellulases even at relatively low concentrations. In addition to complete inhibition by tannic acid, TeCel7A-TrCBM was strongly inhibited by monomeric phenols such as cinnamic acid, coniferyl aldehyde, 4-hydroxybenzaldehyde and syringaldehyde. Increasing concentrations of coniferyl aldehyde and syringaldehyde decreased TeCel7A-TrCBM activity during the 24 hr incubation period. TrCel5A was strongly inhibited in the presence of tannic acid and complete inhibition was observed in the presence of HMF. The other inhibitor compounds (except for vanillin and syringaldehyde) strongly inhibited TrCel5A without deactivating the cellulase. Phenolic compounds, coniferyl aldehyde and syringaldehyde showed a substantial deactivation of the cellulase at higher concentrations, but not at lower concentrations. Our results suggest that cellulases could tolerate compounds containing an aldehyde functional group in hydrolysate at lower cellulase concentrations. However, when the concentration of these compounds increased in the hydrolysate, they deactivated the cellulases. The increased concentration of compounds containing an aldehyde group also increased the

hydrophobic environment, which hinders productive binding of the cellulases to the substrate. This knowledge could be beneficial in selecting pretreatment strategies that do not release high concentrations of hydrophobic pretreatment by-products. In future studies, the performance of cellulases in the degradation of hyrolysates pretreated differently to yield by-products with varying concentrations of hydrophobic compounds, should be evaluated.

Enzyme binding to cellulose substrate is not always productive due to a number of substrate and enzyme-related obstacles that halt the enzymatic hydrolytic action (Varnai et al. 2011). In this study, a detailed investigation of enzyme-related obstacles (cellulase-inhibitor compounds interaction) provided a better understanding of the impact of individual inhibitor compounds on enzymatic hydrolysis as opposed to a mixture of inhibitor compounds in hydrolysates. Some enzyme properties that facilitate productive adsorption to cellulose substrates can also contribute to their non-productive binding to lignin. This binding is proposed to be facilitated mainly by hydrophobic forces and to a limited extent by electrostatic interactions and hydrogen bonding (Rahikainen et al. 2013). The observations from this study were in agreement with this proposal, as hydrophobic binding was observed to be the main interaction in the presence of when furans and/phenolic compounds, but no in the presence of only weak acids.

The differences observed in the inhibition of cellulases by inhibitor compounds confirmed that binding affinities varied for different enzymes and different compound characteristics. The impact of selected inhibitor compounds on the hydrolysis of Avicel was also investigated using a combination of TeCel7A-TrCBM and TrCel5A with the addition of Novozyme 188 β-glucosidase to prevent feedback inhibition by cellobiose. In a complex cellulase system, acetic, tannic and formic acids were observed to have strong inhibition effects. The inhibition effect on a complex cellulase system suggested that the weak acids and tannic acid were not only a problem for individual cellulases. Many studies on unproductive adsorption of cellulases to cellulose have been conducted on crude commercial enzyme mixtures or partially purified commercial enzyme mixtures, where impurities such as stabilizers may impact the results (Zheng et al. 2013). The approach to use individual recombinant enzymes offers a cheap alternative to commercial enzymes.

We investigated the mechanism of enzyme and inhibitor binding by using furfural, syringaldehyde, coniferyl aldehyde and vanillin as representative furan aldehydes and phenolic compounds (Chapter 5). A competitive binding to hydrophobic residues exposed on the surface of the protein was observed in the presence of both the selected inhibitors and the fluorescence dye. The inhibitors bound to the exposed residues competed with the fluorescence dye and thus reduced the fluorescence intensity. Most cellulases have domains with hydrophobic amino acids exposed on the surface, for instance TrCel7A has three tyrosine residues exposed in the CBM domain and a tryptophan residue exposed in the catalytic domain. These hydrophobic residues are positioned for specific and optimal interaction with the cellulose substrate (Eriksson et al. 2002). The exposed amino acids on *T. reesei* enzyme surfaces also increased protein hydrophobicity and enhanced binding to lignin residues in lignocellulose material (Palonen et al. 2004). We noted that some cellulases (TeCel7A-TrCBM, TrCel5A and SfCel3A) retained most of their activity in the presence of furfural and monomeric phenols such as syringaldehyde, vanillin and coniferyl aldehyde. However, inhibitors such as coniferyl aldehyde, vanillin and syringaldehyde only competed with the binding of the fluorescence dye to the protein, without a significant effect on the stability of the protein. This could mean that although the furan aldehydes and monomeric phenols were bound to the protein, their binding had a minimal effect on the activity and the stability of the protein.

Hydrophobic interactions between cellulases and phenolic structures in lignin residues are understood to be the main mechanism facilitating unproductive binding (Varnai et al. 2014). This mechanism is different from specific targeted binding of cellulases to cellulose typically mediated by a cellulose binding module (CBM) of the cellulase (Zheng et al. 2013). The CBM is also proposed to enhance non-productive binding of enzymes to lignin residues that triggers irreversible enzyme adsorption (Gao et al. 2014). Currently, there is no direct evidence to support this hypothesis, but a number of studies reported that cellulases containing a CBM have increased affinity for lignin compared to CBM-less cellulases (Vermaas et al. 2015). However, we observed that the presence or lack of a CBM moiety in TeCel7A had no substantial impact on binding of the protein to inhibitor compounds. The *T. reesei* CBM used in this study belongs to family 1 CBMs and is only about 5 kDa in size, while the catalytic domain is about 55 kDa. It has been reported that the role of smaller CBMs in cellulase-lignin-binding may be insignificant if the catalytic domain is large and capable of binding to lignin (Rahikainen et al. 2013). This might have been the reason why there was no difference observed in the binding of a TeCel7A with or without a TrCBM.

Non-productive adsorption of proteins to inhibitor compoundschanges their conformational structure and intensifies the protein-surface interaction, which could lead to the denaturation of cellulases (Vollrath et al. 2014). Poor thermal stability of enzymes also contributes to denaturation and is driven by protein aggregation and precipitation, which results in reduced soluble protein concentration and lower net recoveries. Rahikainen et al. (2011) reported that cellulase-lignin interactions were more pronounced at temperatures from 45°C and higher, thus suggesting that protein unfolding facilitated by an increase in temperature may cause the protein to lose its structure and become irreversibly bound to lignin surfaces. A possible means of avoiding protein denaturation at elevated temperatures during hydrolysis, is to reduce the processing temperature or to use surface coating agents such as non-ionic surfactants. However, higher temperatures are desirable in industrial systems since they improve heat transfer and reduce processing times. Increasing the process temperature also lowers the viscosity of the system and may lead to higher specific activities, which are desirable for overall performance (Hall et al. 2011).

The use of surface coating agents such as non-ionic surfactants or polymers has been widely investigated (Borjesson et al. 2007, Sammond et al. 2014, Eriksson et al. 2002). These strategies could be applied in diluted hydrolysates, but they are limited in hydrolysates containing high concentration of inhibitor compounds. Enzymatic hydrolysis of strongly inhibiting lignocellulosic hydrolysates can be dramatically enhanced by the application of detoxification (or conditioning) strategies. The drawback of detoxification is often cited to be a requirement of a separate process step that has economical implications. However, recent developments showed that detoxification can be applied *in situ* using reducing agents under mild conditions (Cavka et al. 2011). The application of sodium dithionite and sodium sulfite improved the enzymatic hydrolysis and fermentation of spruce and sugarcane bagasse hydrolysates (Cavka et al. 2011, Cavka and Jönsson 2013). We observed that detoxification with sodium sulfite and/or laccase showed significant improvement in alleviating coniferyl aldehyde inhibition. Syringaldehyde did not result in significant inhibition of the enzymatic hydrolysis under the conditions tested, but the addition of sodium sulfite and/or sodium dithionite to reactions with syringaldehyde resulted in a decrease in glucose production. It was also interesting to note that detoxifying agents added to pure cellulose in the absence of inhibitors resulted in the inhibition of enzymatic hydrolysis reactions. This suggests that the detoxifying agents bind to inhibitors in the reaction and

prevent these inhibitor compounds from binding to cellulases. However, in the absence of the inhibitors in the reaction, the detoxifying agents might bind to the cellulases and prevent efficient hydrolysis. This means that the addition of detoxifying agents is only beneficial if inhibitors are present in a hydrolytic system.

The mechanisms and theories deducted from the interaction of individual cellulases may not be displayed in enzyme systems where cellulases are in a mixture due to a more complex and competitive binding of multiple enzymes competing for the substrate (Yarbrough et al. 2015). The interaction between inhibitor compounds and enzymes depends on multiple factors based on the surface properties of the protein and inhibitor compounds (e.g. lignin), therefore a simple and direct relationship is not obvious. The relationship is further complicated by varying structural and chemical properties of inhibitor compounds inherent to the feedstock and is also altered by the severity and chemistry of the pretreatment process. The affinity of the enzyme for a specific substrate is also influenced by its physicochemical properties such as molecular weight, surface amino acid charges, hydrophobicity and intermolecular interactions such as hydrogen bonding (Yarbrough et al. 2015. The dynamic changes in substrates as the hydrolysis advances further contribute to the complexity of the substrate.

6.2 CONCLUSIONS

The work presented in this study has covered the main objectives stated for this study. The highlights of the research include the following:

- Cellulases were inhibited/deactivated differently by the inhibitor compounds and this effect largely depended on the specific interaction between the particular cellulases and the inhibitor compounds.
- The dominating mechanism for furan aldehydes and phenolic compounds was hydrophobic interactions, whereas ionic interactions dominated for weak acids.
- Hydrophilic compounds such as hydroxybenzoic acid did not significantly inhibit any of the cellulases investigated, whereas the more hydrophobic compounds such as tannic acid had damaging effects.
- A strong inhibition effect in the presence of weak acids was only observed on the retaining enzymes (SfCel3A and TeCel7A-TrCBM) and not on the inverting enzyme

(TrCel5A), which suggests that weak acids affected a double displacement retaining mechanism in glycoside hydrolases.

- The detoxification strategies revealed that hydrolysis could be slightly increased by the application of sulfur oxyanions (e.g. sodium dithionite). Sulfur oxyanions was observed to detoxify the inhibiting compounds in the hydrolysis reaction. However, the addition of detoxifying agents inhibited the hydrolysis reactions in the absence of an inhibitor compound in the reaction.
- An interaction between the aldehyde inhibitors (syringaldehyde and coniferyl aldehyde) and detoxifying agents (sodium sulfite and sodium dithionite) was observed, but this did not improve glucose production.
- There was a significant loss in TeCel7A-TrCBM residual activity in reactions containing coniferyl aldehyde, with no improvement when a detoxifying agent (sodium dithionite or sodium sulfite) was added.
- A decrease in TeCel7A-TrCBM residual activity in the presence of syringaldehyde was also observed, however, an improvement in residual activity was observed in the presence of the detoxifying agent.
- The effect of detoxifying agents (sodium sulfite and sodium dithionite) was more profound with soluble inhibitors in the liquid hemicellulose fraction or individual inhibitor compounds than with insoluble inhibitors in the filter cake.
- We speculated that acetic acid exposed the buried hydrophobic regions in the destabilised cellulase, but did not bind to the exposed hydrophobic regions. This further proved that weak acids may not be involved in hydrophobic interaction, but may rather bind to the cellulases through ionic interactions.
- Furan aldehydes and phenolic compounds were observed to bind to the cellulases through hydrophobic interactions, but we found that the CBM had no significant effect on the hydrophobic binding.

This study revealed that there was a selective inhibition/deactivation effect that depended mainly on the chemical characteristics of both the cellulase and the respective inhibitor compound. Furthermore, phenolic compounds and furans interacted with cellulases mainly through hydrophobic interactions, while electrostatic interactions were the main mechanism for weak acids. This can be investigated further in future, which could include the inhibition effects of weak acids at varying pH values. Future studies should also investigate the effects of adding accessory enzymes together with cellulases in the detoxification reactions. The mechanisms behind the interaction of some cellulases and selected inhibitor compounds

could not be defined in this study, such as the enhancement of endoglucanase activity in the presence of HMF. Future studies could also focus on establishing a specialised technique for studying these mechanisms.

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Appendix: Additional file 1: Residual % activity of cellulose enzymes

Table 1 **Inhibition:** Residual % activity of SfCel3A enzyme (pNPG substrate). Results represent the mean value of three repeats and standard deviations are indicated with ±.

Inhibitor	% Inhibitor concentration			
	25%	**50%**	**75%**	**100%**
Acetic acid	68.0±5.4	35.4±0.95	26.3±2.5	13.4±3.34
Formic acid	23.8±2.0	9.10±9.4	1.60±0.6	0
Furfural	100±5.4	102±14.2	105±11	98.9±0.63
Cinnamic acid	102±4.5	110±0.8	110±3.2	118±0.5
Hydroxybenzoic acid	102±5.3	103±6.5	101±5.1	104±5.3
Hydroxybenzaldehyde	71.5±7.5	103±5.5	110±13	113±11
Vanillin	113±5.9	123±2.1	128±12	103±5
Hydroxyacetophenone	102±5.0	96±7.0	109±2.3	118±3.6

Table 2 **Inhibition:** Residual % activity of BGL1 enzyme (cellobiose substrate). Results represent the mean value of three repeats and standard deviations are indicated with ±.

Inhibitor	% Inhibitor concentration			
	25%	**50%**	**75%**	**100%**
HMF	92.0±4.9	94.6±5.9	88.0±0.4	79.0±2.4
Coniferyl aldehyde	124±13.3	127±5.5	136±7.8	146±7.4
Syringaldehyde	89.8±2.0	89.0±1.9	86.7±5.8	76.8±2.0
Tannic acid	14.5±3.9	11.4±3.8	10.6±0.3	8.40±1.2

Table 3 **Deactivation:** Residual % activity of BGL1 enzyme (pNPG substrate). Results represent the mean value of three repeats and standard deviations are indicated with ±.

Inhibitor	Time (hours)	% Inhibitor concentration			
		25%	50%	75%	100%
Acetic acid	1	52.0±7.8	28.9±3.8	14.8±1.6	5.7±1.2
	6	53.7±4.5	33.0±0.87	16.9±3.1	5.30±1.5
	24	33.8±2.7	20±2.3	9.10±2.7	1.70±0.92
Formic acid	1	15±2.5	0	0	0
	6	8.1±5.1	0	0	0
	24	0	0	0	0
Furfural	1	91.1 ±3.5	98.8±7.5	92.6±3	102±6.9
	6	99.7±4.2	109±5.8	111±4.9	113±12.1
	24	103±0.96	106±0.84	124±15	100±4.7
Cinnamic acid	1	94.0±14	110 ±9.3	129±11	127±6
	6	106±5.1	126±4.9	133±13	135±5.2
	24	103±13	117±5.9	128±5.1	117±16
Hydroxybenzoic acid	1	91.0±2.7	96.0±11	107±5.9	92±8.2
	6	108± (2.3)	116±5.9	122±6.0	119±4.3
	24	92.6± (4.4)	97.7±4.3	112±3.6	99.5±4.2
Hydroxybenzaldehyde	1	122±13.4	130±15	130±12.8	120±10
	6	102±3.1	114±12.9	111±9.3	111±4.5
	24	113±15	108±9.7	115±17	97.0±2.4
Vanillin	1	38±4.8	46.9±4.6	76.7±4.6	80.6± 10.9
	6	66.8±6.2	99.6±9.4	107±6.6	113±7.3
	24	67.7±5.5	69.4±6.1	71.3±10.9	93.6±5.3
Hydroxyacetophenone	1	99.7±1.6	108±16	115± (8.1)	108±7.3
	6	89.6±3.6	93.2±2.7	97± (1.2)	99.0±7.3
	24	112±5.5	114±13	101± (5.3)	105±4.3

Table 4 **Deactivation:** Residual % activity of BGL1 enzyme (cellobiose substrate). Results represent the mean value of three repeats and standard deviations are indicated with ±.

Inhibitor	Time (hours)	% Inhibitor concentration			
		25%	50%	75%	100%
HMF	1	95.3±9.7	95.4±1.1	76.0±12	72.0±1.8
	6	82.8±15	85.6±9.6	71.8±1.3	62±4.3
	24	112±7.7	113±2.8	100±4.6	98.2±3.9
Coniferyl aldehyde	1	93.6±8.4	77.5±3.5	81.9±9.7	51.3±3.4
	6	81.3±12	67.6±4.5	57.9±3.3	41.3±4.0
	24	87.0±3.5	60.0±3.8	55.9±4.6	42.2±3.3
Syringaldehyde	1	89.3±4.2	83±2.0	72.8±3.2	104±2.4
	6	72.6±6.0	57.7±12	49.6±3.7	57.4±6.1
	24	113±9.4	107±16	71.8±8.7	46±6.1
Tannic acid	1	11.7±4.6	7.3±1.1	4.8±2.7	3.6±0.8
	6	5.4±2.6	4.3±0.7	3.3±2.0	2.9±0.6
	24	8.70±2.0	10±0	6.5±2.6	9.9±1.8

Table 5 **Inhibition:** Residual % activity of CBH1 enzyme (pNPC substrate). Results represent the mean value of three repeats and standard deviations are indicated with ±.

Inhibitor	% Inhibitor concentration			
	25%	50%	75%	100%
Acetic acid	89.2±9.6	63.4±9.3	50.1±7	41.5±3.5
Formic acid	43.0±14	51.4±2.4	29.8±8.0	60.3±2.0
Furfural	60.5±12	49.6±14	43.7±10	38.6±11
HMF	44.8±11	51.3±1.5	39.5±8.8	51.0±12
Cinnamic acid	34.4±7.9	17.2±6.9	32.9±15	17.4±4.2
Hydroxybenzoic acid	75.3±7.0	58.5±2.7	60.6±7.5	59.2±11
Hydroxybenzaldehyde	48.9±13	30.2±5.1	52.3±7.6	39.2±9.7
Vanillin	60.7±1.5	47.8±8.3	52.5±1.2	44.6±7.0
Hydroxyacetophenone	65.9±8.2	56.4±10	65.6±8.5	69.3±12

Table 6 **Inhibition:** Residual % of CBH1 enzyme (MULac substrate). Results represent the mean value of three repeats and standard deviations are indicated with ±.

Inhibitor	% Inhibitor concentration			
	25%	50%	75%	100%
Coniferyl aldehyde	58.3±1.6	35.5±0.7	23.0±2.6	15.4±1.3
Syringaldehyde	44.6±2.2	31.1±0.1	19.8±0.9	11.0±0.5
Tannic acid	9.03±2.5	3.7±0.3	3.7±0.1	0

Table 7 **Deactivation:** Residual % activity of CBH1 enzyme (pNPC substrate). Results represent the mean value of three repeats and standard deviations are indicated with ±.

Inhibitor	Time (hours)	% Inhibitor concentration			
		25%	50%	75%	100%
Acetic acid	1	58.9±5.7	70.0±9.7	66.3±1.3	60.1±1.3
	6	63.7±2.7	66.6±5.2	60.9±3.6)	61.0±8.4
	24	74.6±3.9	74.1±3.9	65.1±3.7	60.0±7.8
Formic acid	1	65.5±11	73.0±2.2	74.1±6.3	69.6±3.4
	6	68.2±9.5	75.9±2.7	75.2±11	71.8±1.6
	24	68.1±13	77.9±9.3	73.9±5.6	75.5±10
Furfural	1	62.4±11	61.3±2.9	52.4±13	48.6±8.1
	6	77.1±11	70.2±3.0	73.6±4.0	58.5±4.4
	24	69.2±11	74.9±9.2	63.6±13	58.9±2.9
HMF	1	72.3±5.4	72.4±3.1	65.7±11.8	62.8±7.4
	6	52.4±3.3	56.4±4.3	60.0±12.2	64.6±2.6
	24	94.5±2.8	63.5±2.6	69.6±7.9	57.0±6.4
Cinnamic acid	1	64.0±6.7	71.0±15	65.1±16	77.2±5.3
	6	62.6±13	68.2±13	68.3±10	78.6±7.5
	24	83.8±3.6	89.0±4.1	77.9±8.9	63.5±4.2
Hydroxybenzoic acid	1	85.9±1.6	66.3±3.8	70.4±8.8	75.8±3.7
	6	84.5±5.2	72.5±5.0	72.1±4.3	68.9±6.3
	24	80.6±4.9	87.1±3.5	83.9±3.7	53.4±7.3
Hydroxybenzaldehyde	1	76.9±9.4	80.1±7.1	71.6±12	69.9±7.8
	6	60.9±4.4	68.1±9.0	62.3±6.1	65.7±7.9
	24	74.4±6.2	74.9±1.2	82.4±8.6	79.4±6.5
Vanillin	1	82.4±9.1	71.0±3.4	64.8±1.0	49.1±1.8
	6	70.4±11	66.9±6.3	60.6±9.5	62.9±7.6
	24	80.0±4.0	70.1±1.3	67.5±5.8	71.0±9.7
Hydroxyacetophenone	1	64.9±4.1	68.6±6.3	65.0±12	60.0±8.3
	6	78.1±8.6	71.2±6.4	69.8±4.0	72.1±8.5
	24	72.0±2.0	77.2±7.4	62.3±3.0	70.3±2.5

Table 8 **Deactivation:** Residual % activity of CBH1 enzyme (MULAC substrate). Results represent the mean value of three repeats and standard deviations are indicated with ±.

Inhibitor	Time (hours)	% Inhibitor concentration			
		25%	50%	75%	100%
Coniferyl aldehyde	1	58.3±1.6	35.5±0.7	23.8±2.6	15.4±1.3
	6	47.8±1.0	34.5±2.9	27.2±6.2	17.5±1.3
	24	42.5±2.4	27.9±1.3	18.7±0.1	12.4±0.7
Syringaldehyde	1	51.8±0.9	33.2±0.42	16.9±2.8	10.9±0.3
	6	51±1.9	32.3±0.2	21.8±0.1	15.6±0.5
	24	43.3±0.8	26.5±0.2	17.2±0.3	11.4±1.0
Tannic acid	1	16.6±0.2	7.60±0.4	1.60±0.1	0
	6	15.8±0.9	8.10±0.2	3.70±0.4	1.0±0.2
	24	10.4±0.4	5.0±0.4	1.70±0.1	0.13±0.1

Table 9 **Inhibition:** Residual % activity of EG2 enzyme (CMC substrate). Results represent the mean value of three repeats and standard deviations are indicated with ±.

Inhibitor	% Inhibitor concentration			
	25%	50%	75%	100%
Acetic acid	87.0±4.4	76.0±4.9	55.0±4.7	45.0±15
Formic acid	85.0±6.5	70.0±11	54.9±4.3	49.0±15
Furfural	54.8±8.4	54.9±12	56.0±9.0	34.0±6.5
HMF	44.7±6.5	44.0±5.9	7.50±6.5	0
Cinnamic acid	63.9±1.8	62.0±12	46.8±4.2	46.0±8.2
Hydroxybenzoic acid	62.0±5.7	63.0±16	42.0±4.3	45.5±6.8
Hydroxybenzaldehyde	53.0±8.9	42.0±2.2	44.7±6.3	36.8±4.0
Vanillin	75.0±24	54.0±0.7	50.7±9.2	54.8±3.3
Hydroxyacetophenone	50.8±7.1	43.7±5.7	33.7±2.4	36.0±4.0
Coniferyl aldehyde	60.6±4.7	44.9±9.0	31.6±5.4	32.7±8.0
Syringaldehyde	47.8±4.1	48.8±5.5	48.6±17	53.5±19
Tannic acid	44.9±2.7	38.0±3.7	33.0±5.2	26.5±5.8

Table 10 **Deactivation:** Residual % activity of EG2 enzyme (CMC substrate). Results represent the mean value of three repeats and standard deviations are indicated with ±.

Inhibitor	Time (hours)	% Inhibitor concentration			
		25%	50%	75%	100%
Acetic acid	1	89.6±11	73±8.5	71.8±14	54.8±3.4
	6	84.8±5.5	78.9±5.6	71.5±8.5	54.3±4.6
	24	70.1±1.3	77±4.7	73.7±6.4	71.7±4.2
Formic acid	1	84.9±11	84.9±8.8	55.2±3.9	47.6±9.6
	6	73.8±10	71.2±7.3	58.3±6.6	54±5.2
	24	72.9±11	66.1±6.3	57.9±7.0	58±1.7
Furfural	1	68.4±8.5	54.9±7.0	33.8±6.2	55.4±2.9
	6	61.9±2.5	74.2±2.4	48.9±12	59.8±1.5
	24	70.6±8.4	53.3±8.7	42.9±5.2	58±5.3
HMF	1	75.5±7.4	39.7±10	44.5±5.1	26.2±10.6
	6	76.0±8.5	41.8±8.4	40.6±1.1	28.6±7.0
	24	83.1±10	86.6±6.3	42.4±4.1	30.2±1.5
Cinnamic acid	1	53.3±4.2	60.1±7.6	56.4±6.7	69.5±9.7
	6	58.8±5.3	61.7±7.8	60.7±13	61.6±7.7
	24	73.3±7.5	74.7±9.1	75.6±11	73.3±11
Hydroxybenzoic acid	1	57.5±8.1	40.0±10	67.7±9.4	61.5±8.3
	6	64.5±6.9	52.6±6.4	36±1.8	86.5±9.3
	24	51.8±9.6	67.6±3.7	73.2±9.8	79.6±8.4
Hydroxybenzaldehyde	1	55.5±4.8	47.2±10	50.1±2.4	81.5±8.8
	6	63.9±3.9	50.2±4.4	52.9±5.2	77.6±3.1
	24	75.6±8.4	78.8±3.0	66.3±8.3	73.5±3.9
Vanillin	1	38.0±4.8	46.9±4.6	76.7±4.6	80.6±11
	6	66.8±6.2	99.6±9.4	107±6.6	113±7.3
	24	67.7±5.5	69.4±6.1	71.3±11	93.6±5.3
Hydroxyacetophenone	1	59.3±3.9	61.7±7.6	48.9±4.7	59.5±7.1
	6	62.4±5.6	68.5±4.9	72.1±8.5	76.5±11
	24	76.6±1.1	64.5±4.8	75.9±5.1	95.4±4.3
Coniferyl aldehyde	1	66.5±2.9	65.9±11	89.9±2.2	76.8±7.2
	6	73.8±7.5	64.4±5.7	67.3±4.2	67.6±5.3
	24	66.2±2.4	72.7±6.0	64.0±6.2	84.7±2.2
Syringaldehyde	1	46.8±7.1	61.5±5.7	43±3.1	96.9±6.2
	6	75.9±4.9	89.9±4.6	85.6±11	111±4.6
	24	44.7±3.6	56.3±8.0	77.6±7.4	98.5±6.3
Tannic acid	1	60.6±12	66.0±5.8	55.9±2.4	36.9±1.8
	6	71.0±12	55.1±9.9	49.0±8.3	47.8±5.1
	24	85.5±7.0	74.8±5.8	57.7±9.2	44.2±8.9